Abnormalities of immune complex processing in systemic lupus erythematosus

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Abnormalities of Immune Complex Processing in Systemic Lupus Erythematosus

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Abstract

Heterozygous deficiency of C4A is a common inherited abnormality in patients with SLE who also acquire a reduction of erythrocyte (E) complement receptor type 1 (CR1) numbers. These factors could contribute to disease expression by reducing the efficiency of complement and ECR1 mediated mechanisms for the physiological disposal of circulating immune complexes (CIC). I therefore studied aspects of the physiology of C4A and C4B, the isotypes of the fourth complement component and the mechanisms of reduced ECR1 expression.

C4A binds preferentially to amino groups, while C4B is more efficient in forming ester bonds with hydroxyl moieties. I therefore examined the differential binding of C4A and C4B to SLE erythrocytes *in vivo* and to heat aggregated IgG ICs *in vitro.* My results demonstrate an excess of C4B associated with erythrocytes and a stronger correlation of serum C4A levels with immune complex deposition. These findings reflect factors which may be of importance during periods of disease activity and high CIC levels in SLE. The twin C4 loci are highly polymorphic including null and duplicated alleles. I have studied the serum levels of C4A and C4B amongst carefully genotyped normal subjects and have confirmed observations of the extensive phenotypic overlap between subjects with differing C4 gene numbers. Factors contributing to phenotypic overlap including age, gender, acute phase responses and abnormal serological reactivity were also studied. I also found that the C4B deficient extended haplotype associated with Felty's syndrome encodes expressed homoduplicated C4A alleles, making impairment of CIC clearance an unlikely pathophysiological mechanism for this association.

Erythrocyte complement deposition and CR1 reduction are found in SLE. The presence of antiphospholipid antibodies (aPL) has been correlated with positive direct antiglobulin reactions, as have low CR1 numbers. I therefore studied the possibility that anticardiolipin antibodies (aCL) were associated with CR1 reduction in SLE and found significant negative correlations between aCL levels (IgG and IgM) and CR1 numbers which were not explicable by an association with disease activity. IgM aCL were also associated with E-surface C4 and C3 suggesting that aCL bind directly to erythrocytes. I then showed that aCL are a frequent finding amongst Coombs' positive subjects (suggesting they may be a common anti-erythrocyte autoantibody) and that aPL in these subjects showed increased reactivity with neutral PLs (a component of the E membrane). Observations suggesting direct binding by polyclonal (but not monoclonal) aCL lend further support to the hypothesis that direct E-binding by aPL antibodies contributes to CR1 reduction in SLE.

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Chapter 1

The Physiology of Circulating Immune Complexes (CIC) and Abnormalities in Patients with Systemic Lupus Erythematosus (SLE)

Section 1
Classical Pathway Opsonisation of Immune Complexes

Introduction

Opsonisation by complement is mediated principally by the deposition of C3 and C4 fragments on pathological surfaces ("opsonisation" specifically refers to interactions which increase the efficiency of *phagocytosis*, however, I will use the term to describe other reactions which result in and from the deposition of complement on an activating surface). While classical and alternate pathway activation both contribute to IC opsonisation, classical pathway deposition of C4 is of greater relevance to the work of this thesis and I will therefore omit discussion of the alternate pathway. The complement system is highly evolved to localise activation to pathological surfaces and to avoid the potentially deleterious deposition of active C fragments on host tissue. The existence of cell surface and fluid phase regulatory proteins is thus an important feature of complement physiology. The major classical pathway regulatory proteins are therefore discussed here, along with a description of the catabolism of activated C4 and C3, factors which are of importance in the understanding of immune complex processing.

Nomenclature

I have here retained the original nomenclature for C2 where the larger surface-bound fragment of C2 is designated C2a. For other complement proteins the smaller activation fragments are designated "a", while the larger activated portion is designated "b". For typographical reasons I have not adopted the practice of indicating activated molecules with a line above the character but have tried to make the activation state of the molecule clear in the text.

1.11 Initiation of classical pathway binding and opsonisation by immunoglobulin

Recognition of antigen by antibody leads to classical pathway activation. Aggregated IgG, or the presence of IgM in the surface bound, planar, configuration, is required and activation results from binding of the globular head of C1q to the aggregated immunoglobulin (reviewed in [2-4]). C1q binds by hydrophobic interaction close to the

IgG hinge region in the Cγ2 region of the constant domain of all IgG Fc regions except for the IgG 4 subclass, and to the Cμ3 region of IgM. Attachment of C1q results in the firm binding of the loosely associated $C1r_2s_2$ tetrameric protein complex, in the autocatalytic activation of C1r and in the subsequent activation, by C1r, of C1s . The association of C1q and $C1r_2s_2$ is calcium dependent and is abrogated by calcium chelators such as ethylenediaminetetraacetic acid (EDTA).

Immunoglobulin class and isotypes have marked effects on classical pathway activating abilities. Human IgG1, IgG3 (when aggregated) and IgM are potent classical pathway activators, while IgG4 and IgA are not (reviewed in [5]). Species differences also exist, mouse immunoglobulins show marked differences in their ability to activate the classical (and alternative) pathway, and in the C solubility of ICs containing different murine immunoglobulins [6].

1.12 Activation of C4 by C1s

Activated C1s has serine esterase activity and is able to cleave a 77 amino acid activation fragment, C4a, from the carboxyterminal of the C4α chain (figure 1, overleaf). Liberation of this fragment induces a conformational change which is associated with the activation of the C4 intrachain thiolester bond (see chapter 1, section 2). The larger activation fragment of the C4 molecule (C4b) is then able to bind to nucleophile groups in the vicinity via the reactive carbonyl moiety of the activated intrachain thiolester bond. The rapid hydrolysis of C4b with tissue water inactivates the intrachain thiolester for covalent binding and places a powerful time, and thus spatial, constraint on the diffusion of the active fragment. By analogy with hydrolysis of C3, the lifetime of activated C4 may be in the order of 60μsec during which time it may diffuse around 40nm. While the cleavage of C4 by C1s is an amplification step, the fixation of C4 to surfaces is inefficient (<5% is deposited) and it is estimated that approximately 30 molecules of C4 attach for each C1s.

1.13 The formation of C3/C5 convertase and C3 activation

C4 functions as a matrix protein, localising complement activation to surfaces previously recognised by IgG and bearing the activated C1 complex. As will be discussed (chapter 1, section 2), the susceptibility of the C4 intrachain thiolester to nucleophilic attack has an important role in determining the efficiency of complement localisation to differing surfaces. Once deposited, C4b forms a complex with nascent C2 and Mg++, which localises C2 for hydrolytic activation to C2a by release of the smaller C2b fragment. Activated C1s is also responsible for the activation of C2 and is capable of activating both fluid phase and surface bound C2. .

Figure 1 : The structure, activation, nucleophile binding and Factor I mediated catabolism of C4

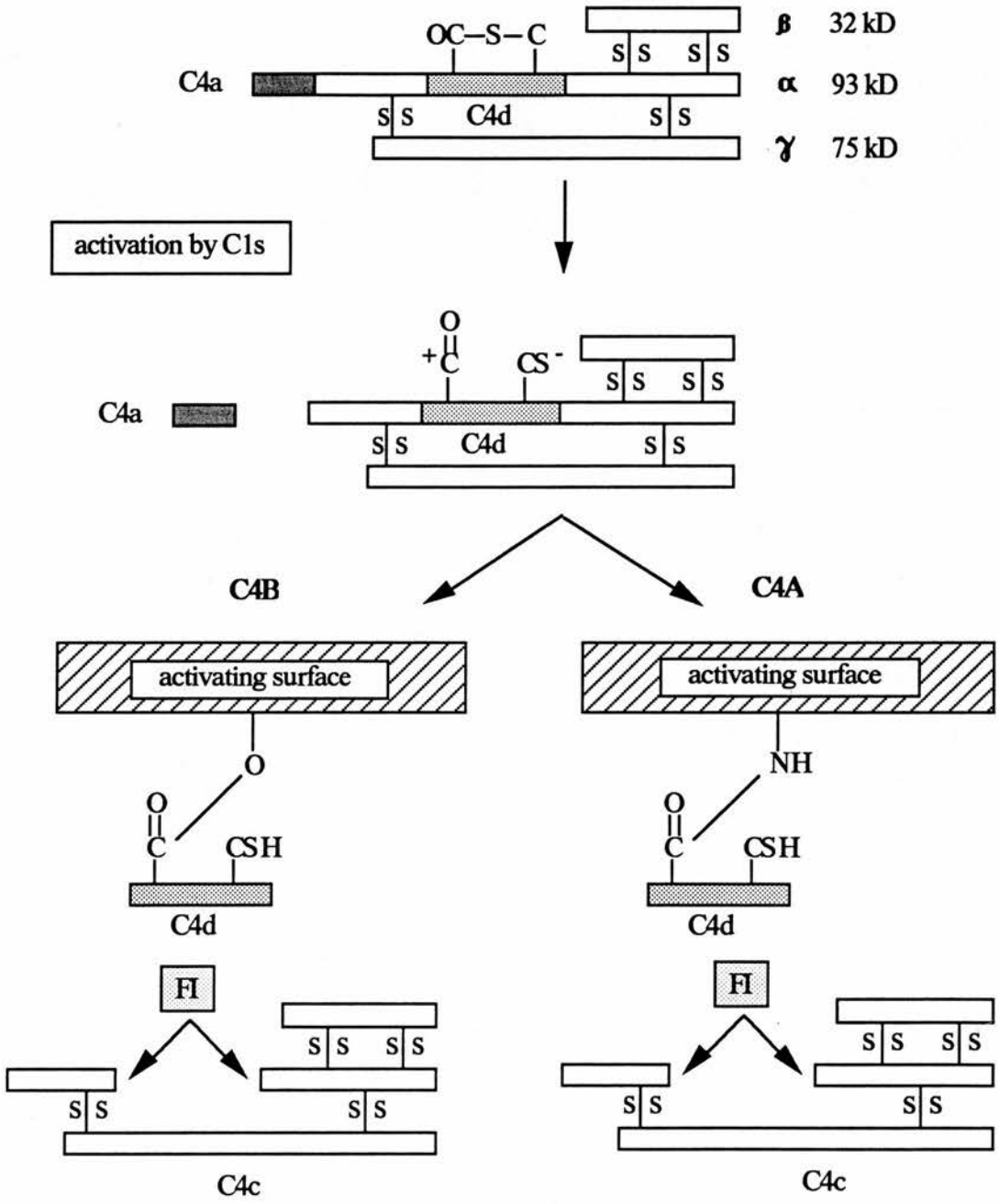

Figure 1: Diagrammatic structure of C4 showing the changes that occur on activation by C1s with the liberation of C4a and the activation of the intrachain thiolester bond in C4b. Covalent binding in ester linkage favoured by C4B is illustrated on the left, while the opposite amide linkage formed preferentially by C4A is on the right. Factor I cleaves the C4 alpha chain in 2 places liberating the larger C4c fragment and leaving the C4d region covalently bound to the activating surface.

However, only C2a which is formed *in situ* in association with C4 has serine esterase activity and is capable of forming the C3/C5 convertase, fluid phase C2a has no catalytic activity. Only C42 molecules within the molecular radius of the C1 complex (approx 60nm) can be activated, a further factor restricting C activation. The active catalytic site of C2a so formed is then able to cleave C3 by the liberation of the 77 amino acid C3a from the NH2 terminal of the C3 α chain in a manner analagous to the activation of C4. C3a diffuses from the site and acts as an anaphylotoxin. Like C4, proteolytic activation of C3 results in formation of an active carbonyl residue in the intrachain thiolester bond which is transiently able to covalently bind C3 via a transacylation reaction with nearby surface-nucleophile groups (figure 2, overleaf). Activated C3b becomes surface-bound and associates with C42 to form the C5 convertase, acting as the site of attachment of C5 for cleavage by activated C42.

While C3 activation is an amplification step, constraints similar to those described for C4 apply and the diffusion distance/binding of C3 is limited by rapid hydrolysis of the intrachain thiolester by tissue water. Approximately 200 C3 molecules may be deposited/C42 complex and under optimal circumstances it may be possible to generate some 100,000 surface bound C3 molecules/sheep E in 1 minute. However, C3 activation is generally less efficient. Estimates of 2000-4000 C3/sheep E for phagocytosis and 5000/E for lysis give an indication of the numbers of molecules required for functional interactions. The subsequent binding of C5 to surface bound C3 and its activation by C2 with liberation of the 74 amino acid C5a anaphylotoxin is analogous to the activation of C4 and C3

1.14 Classical pathway regulatory proteins

While essentially an amplifying cascade, the process of classical pathway activation may be seen to incorporate a number of features which act to limit C deposition to activating surfaces. The requirement for polymerised IgG or IgM in the staple conformation, the requirement for both C2 and C5 to be bound to C4 and C3 on the activating surface for convertase activity, the steric limitation of C1s and the short half-life of the activated thiolester of C4 and C3, may all be considered to reduce the possibility of heterotopic complement activation. However, in addition to these factors, a number of specific cell surface and fluid phase regulatory proteins exist.

Figure 2: The activation, surface binding and catabolism of C3

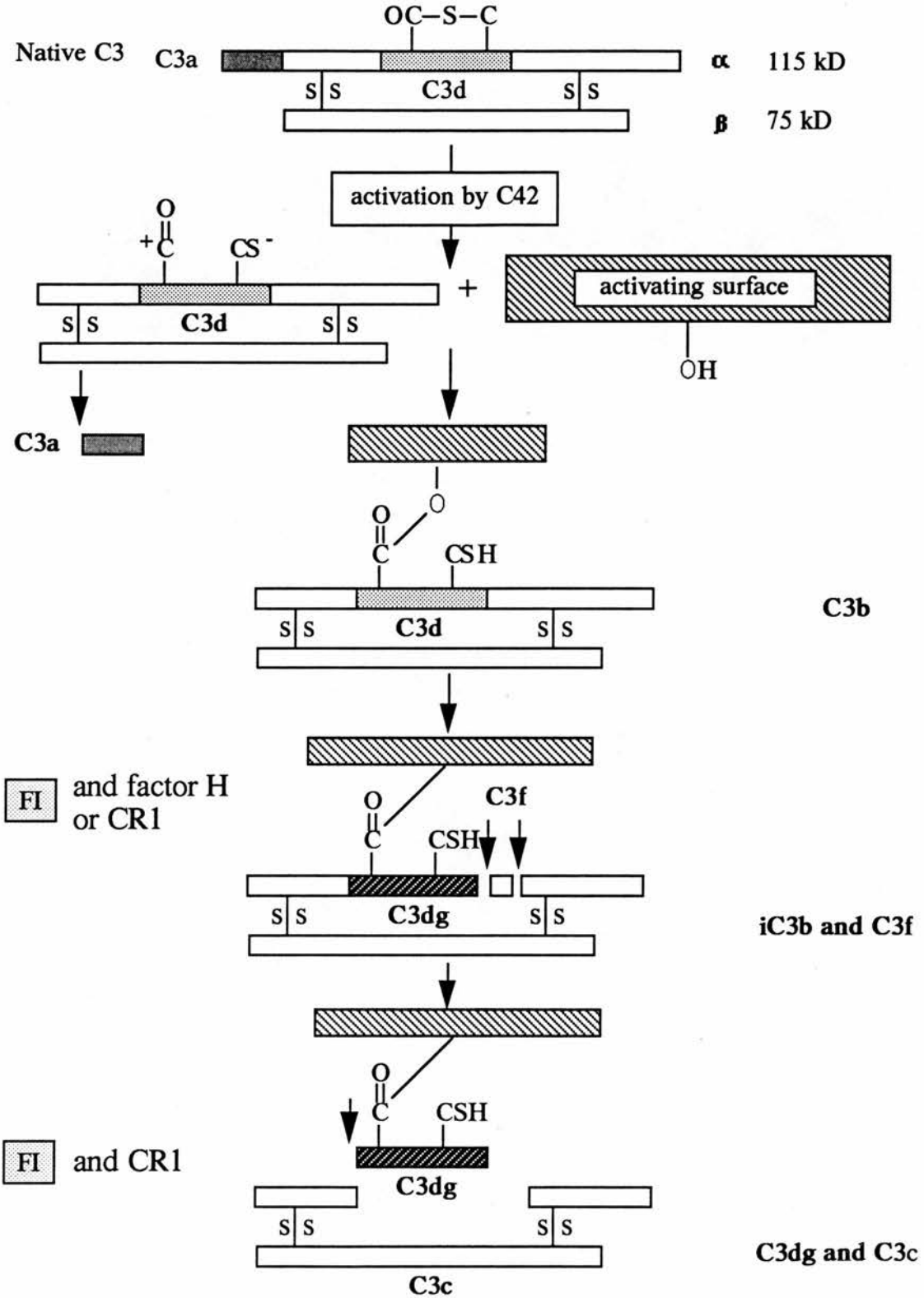

Figure 2: Diagrammatic structure of C3 showing the formation of the active intrachain thiolester bond after cleavage by C42. The designation of intermediary and catabolic fragments of surface bound C3 is given in bold type, while the fluid phase regulatory proteins Factor I (FI), Factor H and complement receptor type 1 (CR1) are shown on the left opposite the C3 fraction they act upon

C1 inhibitor (C1-INH) is a heavily glycosylated non enzymatic inhibitor of activated C1, which also functions as a specific inhibitor of kallikrein, plasmin and clotting factors IX and XII. One -to-one binding of C1-INH to the active catalytic sites of C1r and C1s results in irreversible inactivation of these molecules, consuming Cl-INH in the process. The presence of C1r-C1s-C1-INH2 complexes in the serum can therefore be used as an indicator of complement activation. C1-INH binding is rapid, and active C1s may have a half-life of <20s at physiological temperatures. Indeed, the whole process of complement activation of test immune complexes in serum has probably ceased after 3 minutes *in vitro.*

Inactivation of the C3/C5 convertase takes place in two phases. The first, acceleration of the autocatalytic dissociation of C2 from C4 is accomplished by two proteins. C4 binding protein (C4bp) is a fluid phase spider-like regulatory protein which binds to C4 near the C2 binding site and can bind up to 6 C4 molecules simultaneously. Decay accelerating factor (DAF) is a membrane glycoprotein molecule which also acts to accelerate the dissociation of C2 and will be described in detail below. The second phase, catabolism of membrane bound C4b is accomplished primarily by Factor I, with C4bp and CR1 as co-factors.

Factor I is the principal fluid phase regulator of complement activation and is an 80kD glycoprotein present in serum at about 30-50μg/ml. In the presence of cofactors, I cleaves C4 at 2 sites in the α chain liberating the larger C4c fraction and leaving the thiolester containing C4d region bound to the surface. In the absence of cofactor activity, only one site is cleaved leading to the inactivation of C4b and leaving the partially catabolised fragment (iC4b) *in situ.* In this instance, there is no dissociation into C4c and C4d (see figure 1). The inactivation of C3b take place in a similar fashion. In the first step either Factor H (a 150kD glycoprotein formerly termed ß1H) or CR1 may function as cofactors, in the second CR1 is the sole cofactor. The initial step is the binding of H or CR1 to the C3b molecule followed by cleavage of the α chain at 2 sites leading to the liberation of a small fragment (C3f) and the formation of iC3b, which remains membrane bound. In the presence of CR1 full catabolism of iC3b occurs by a 3rd cleavage in the α chain which releases the larger part of the molecule as C3c, leaving C3dg bound to the activating surface. The activation and catabolism of C3 is illustrated in figure 2.

DAF is a 70kD cell surface protein which shares an unusual method of membrane attachment with a number of other molecules including acetyl cholinesterase (AChE), alkaline phosphatase, 5´ nucleotidase and a trypanosome surface glycoprotein [7]. The molecule is attached to the membrane by a phosphatidylinositol group, which is itself linked to the functional moiety by a "tail" of amine and oligosaccharide groups. Medof and

colleagues [8] noted that purified DAF was able to re-incorporate into the erythrocyte membrane with retention of functional activity. This unusual property is ascribed to the nature of the membrane attachment group. In addition to C42 decay accelerating activity, Medof noted that DAF acted prior to this step to inhibit the incorporation of C2 to membrane bound C4. Their studies suggested that as few as 70 DAF molecules could prevent C mediated lysis of sheep E. By comparing the effects of cell surface DAF and CR1 on C molecules on neighbouring cells, the conclusion was reached that DAF can only act on C localised to the "parent " cell surface while CR1 may also act extrinsically, on nearby cells. These studies emphasise the protective effect of endogenous DAF which is present on all human cell membranes and is deficient (along with AChE and other cell surface proteins) in the clonal disorder paroxysmal nocturnal haemoglobinuria (PNH) [9]. Patients with this disorder suffer C-mediated intravascular haemolysis.

1.15 Bystander C deposition, C binding to immunoglobulin and non Ig dependent C activation

The foregoing concentrates on the deposition of C proteins on activating (cell) surfaces via aggregated immunoglobulin and the classical pathway, however, a number of other mechanisms may operate in defined situations. Cell surface complement deposition may be secondary to the fluid phase activation of C as a "bystander" phenomenon, without the cell having taken part directly in immune complex formation. The cellular "processing" of ICs, principally by the erythrocyte CR1 mechanism may also lead to the secondary cell surface deposition of C3 and C4 fragments without the erythrocyte having been directly involved as antigen (see chapter 1, section 5).

Complement may bind directly to the variable region of the heavy chain of complexed IgG [10], rather than the cell surface. Immunoglobulins are known to differ in their C activating properties on the basis of isotype and species and differences between isotypes are found at the level of C1q binding and subsequent C4 activation and surface attachment [11]. In general, C activation is independent of Ig binding specificity (eg monoclonal myeloma proteins), but in some cases the nature of the antigen may also be important. Using monoclonal IgM rheumatoid factor from cryoprecipitate of patients with essential mixed cryoglobulinaemia, Ng and colleagues [12] demonstrated that ICs of these antibodies and IgG were able to activate complement in the fluid phase, but did not themselves fix C and did not bind to erythrocyte CR1. Given the importance of rheumatoid factors in rheumatological disease, such details of complement physiology may be of pathological significance. Taylor and co-workers have measured the deposition of C3b on soluble IgM DNA/anti-dsDNA complexes [13]. They noted these complexes fixed little C3b, and that the majority bound to the IgM antibody. E-CR1 binding by these ICs was

correspondingly low. These findings were considered to emphasise the potential importance of characteristics of the antigen, particularly DNA, in IC physiology.

A variety of antibody-independent mechanisms of classical pathway activation have also been described, for example with group B Streptococci [14] and Cardiolipin (CL) [15]. This latter mechanism was critically dependent on CL and when present at a critical density on artificial liposome surfaces, CL bound C1q sufficiently strongly to resists C1-INH inhibition of C1rs incorporation. Alternatively, plasma and bacterial enzymes are capable of inducing formation of a classical pathway C3 convertase from fluid phase C4 and C2 without the mediation of aggregated immunoglobulin or C1 [16]. An autoantibody derived from SLE B cells has been shown to stabilise C42, the classical pathway C3 convertase, analogous to C3 nef [17] and may contribute to hypocomplementaemia in some patients. A recent study purports to show a disease specific deficiency of C4 function (in insulin dependent diabetes) [18]. However, the function tested was sheep cell haemolysis and the differences between disease and control groups observed could readily be due to unequal inheritance of C4A (or C4B null alleles) Differences in C4 haemolytic function were not noted amongst disease discordant monozygotic twins studied.

Section 2

C4A and C4B, the Isotypes of the Fourth Component of Complement: Molecular Genetics, Structure, Serological Reactivity and Function

Introduction

The isotypes of C4, C4A and C4B are highly homologous proteins which appear to have arisen by gene duplication with putative gene conversion events contributing to the high degree of polymorphism which characterises this genetic region. Despite their similarity, important physiochemical and functional differences exist between them and may contribute to the association of genetically determined deficiencies of C4 isotypes with a number of diseases including SLE (see chapter 1, section 3). A variety of polymorphisms are exhibited by C4 including extensive allotypic variation and polymorphism of gene number and size. In addition, C4 proteins exhibit a number of serological markers which have a complex relationship to the genetically defined molecular sequence, specifically in that part of the C4d region which determines the isotype and contains the intra-chain thiolester bond. I will here describe important features of the structure, function, serological reactivity and molecular genetic characteristics of these isotypic proteins.

1.21 The organisation of the human MHC and polymorphism of class III genes

C4A and C4B are encoded by highly polymorphic tandem loci in the class III region of the MHC cluster on the short arm of human chromosome 6. Along with these C4 genes and their associated steroid 21 hydroxylase (21OH) loci, the class III region also encodes C2 and Factor B (Bf) A detailed molecular map of this region, based on the alignment of 4 overlapping cosmid clones of cDNA has recently been published [19]. The overall layout of the MHC is illustrated in figure 3 (overleaf). The entire MHC is thought to be encoded by approximately $2x10^6$bp, of which a 98kb segment comprises the class III region. The Bf and C2 genes lie 2kb apart and are about 30kb from the duplicated C4 and steroid 21OH genes, themselves separated by 10kb. Human C2 and Bf have probably arisen as a duplication from a common ancestral gene and their genetic structure appear to be phylogenetically stable (reviewed in [20]). In contrast, murine and human C4 are highly variable, although a greater degree of conservation is observed around the thiolester binding site in the mouse and human C4α chain and in the γ chain where homology is 93%.

Figure 3. Diagrammatic map of the human MHC and class III region

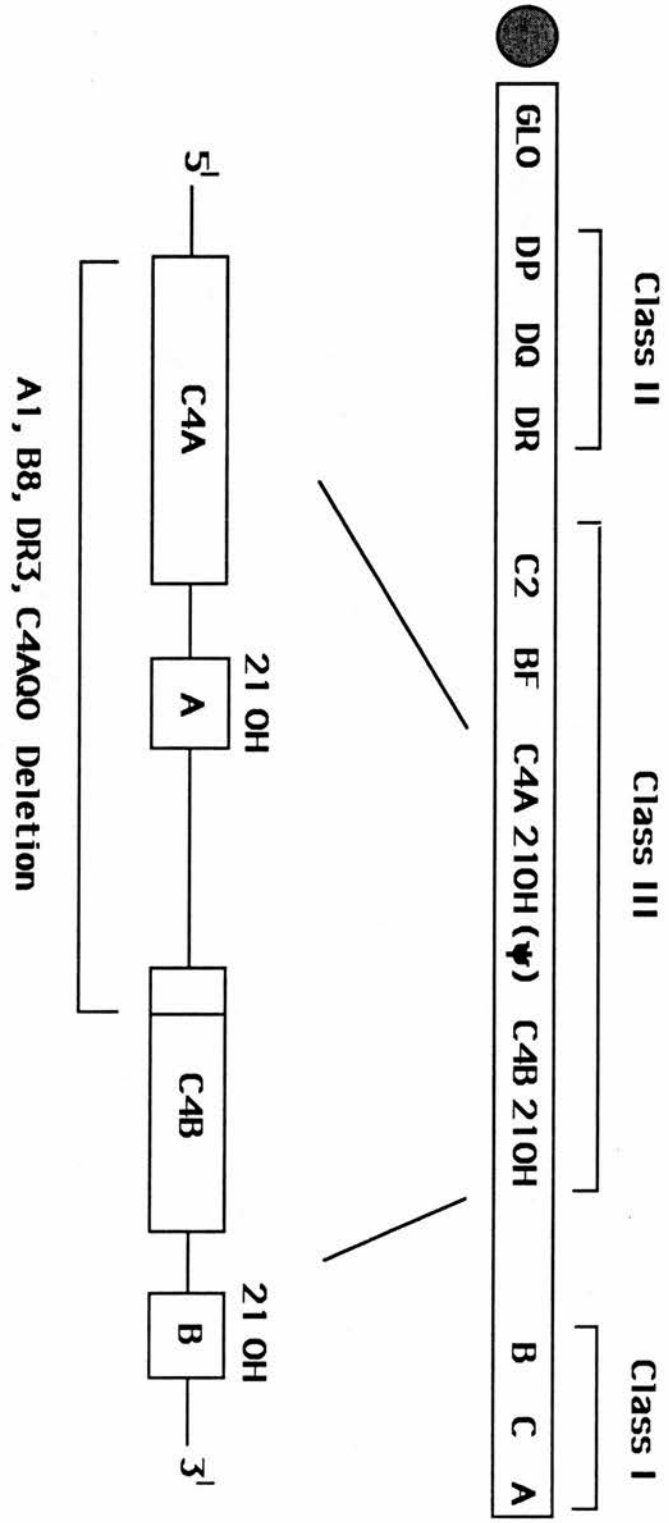

Figure 3. Diagrammatic map of the human MHC and class III region. For legend see text.

Ford used the term "genetic polymorphism" in 1942 [21] to describe the appearance of structural genetic variants at a frequency too great to be explained by mutation alone, and there is usually assumed to be a functional difference between the polymorphic variants (reviewed ref [20]). These differences are thought to underlie the genetic pressure which conserves the polymorphism, though a similar effect would result from linkage of the polymorphic site with a second locus. Functional properties such as haemolysis may be utilised in C4 allotyping and electrophoretic recognition of C4A and C4B allotypes is based on the technique of high resolution agarose gel electrophoresis of neuraminidase treated plasma followed by haemolytic overlay in agarose [22,23]. An improvement in this technique based on Carboxypeptidase B treatment of plasma has recently been reported [24]. Using these techniques, serological typing [25-27] and direct DNA sequencing [28-30] >40 C4 alleles have been recognised.

1.22 C4: structure, activation and internal thiolester bond

Human C4 is a 200Kd molecule synthesised as a single polypeptide chain and split posttranslationally by a plasmin-like enzyme into three disulphide-linked chains (α,β,γ) of 93, 75 and 32Kd respectively (figure 1) (reviewed in [31]). Electron microscopy reveals that C4 is an irregular, globular protein.

C4, along with C3 and α-2 macroglobulin, shares a conserved region of 9 amino acids which encodes a reactive intrachain thiolester bond. This is located at residues 991-994 in the C4 α chain and comprises a glutamic acid residue whose COOH moiety is bound to the SH of a neighbouring cysteine. Activation of nascent C4 to C4b results from the C1s mediated proteolytic cleavage of the 77 amino acid fragment C4a from the N terminal end of the α chain. Upon activation, a conformational change takes place in the molecule (see below) which contributes to the reactivity of the intrachain thiolester bond responsible for C4 covalent surface binding. Specifically, a reactive carbonyl (acyl) group is formed and becomes receptive to transacylation reactions with nearby surface bound nucleophilic proton donors. The activated thiolester bond of C4b (and C3b) can form either amide bonds with amino groups or ester bonds with hydroxyl moieties [32-34].

1.23 C4A and C4B: differential reactivity of the intrachain thiolester

Initial observations of the functional characteristics of the electrophoretic bands of C4 [35] were followed by the recognition that C4B (the basic allele) was more efficient in mediating haemolysis of sheep erythrocytes in the haemolytic overlay assay [22,36]. Differences in immune haemolysis reactivity could arise at several stages including: the activation of isotypes by C1s, the efficiency of thiolester to surface-nucleophile transacylation, binding

and activation of C2 to form an efficient C3 convertase and susceptibility of the C42 molecule to inactivation by regulatory proteins. Law, Dodds and Porter [37] and Isenman and Young [38] have been principally responsible for elucidating the basis of the differential functional capacities of C4A and C4B.

Isenman and Young performed an extensive series of experiments using C4 isotypes purified from Chido and Rodgers negative sera. Their results confirmed that C4B was more efficient than C4A in the sheep erythrocyte haemolytic assay, with a ratio of C4B/C4A activity of 4:1. This was due entirely to greater binding of C4B to the erythrocyte surface and no differences were noted between C4A and C4B in the rate of activation by C1s or in the formation or decay of an active C3 convertase. Treatment of nascent C4b with methylamine was used as a test of the overall reactivity of the activated thiolester bond and no difference between the isotypes was noted. The nature of the covalent bond linking C4 to the erythrocyte surface was examined. Hydroxylamine at alkaline pH readily cleaves ester but not amide linkages. It was found that hydroxylamine treatment of EC4 ghosts resulted in the liberation of 80% of C4B but <20% of C4A implying that the greater efficiency of C4B was due to the formation of ester linkages with the erythrocyte surface, while C4A forms amide bonds. This was further confirmed by observing the potent inhibition of ^{125}I-C4A binding to E by prior fluid phase reaction with the small the amino groups lysine and glycine, while sugar molecules were more effective in inhibiting C4B binding.

The importance of these studies lies not only in defining the nucleophile preferences of the thiolester bond in nascent C4b, but also in suggesting that the reduced haemolytic activity of C4A may be related to the characteristics of the activating surface nucleophile groups and not to functional impairment of C4A itself, *i.e.*that C4A is not a hypofunctional C4 molecule.

Law, Dodds and Porter examined the haemolytic activity of sera from donors of known C4 genotype with sheep E. Though there was considerable variation in the haemolytic activity noted, the ratios of activity/μgC4 in serum are very close to 3 for C4B only, 2 in mixed sera and 1 in the presence of C4A alone. These ratios appear to argue against competitive interaction of C4 isotype binding. Binding of an equimolar mixture of purified C4 isotypes to haemolysin coated E and to BSA/anti-BSA immune complexes was quantified by radioligand binding with ^{125}I-F(ab´)2 anti C4. A maximal two-fold excess of C4B binding to erythrocytes was found, while C4A was more efficient in binding to the protein antigen, though only 30% excess C4A binding was detected. These authors also report that C4 activity is stable in buffer at 37°C for up to 77hrs and independently confirmed the results

obtained by Isenman and Young with regard to small molecule (glycerol/glyceride) transacylation preferences.

Further studies reported by Isenman and Young [39] point to differences between human and sheep cells in the binding of C4 isotypes. The ratio of deposition of C4B/C4A was again found to be around 4:1 for sheep EA, but fell to not more than 2:1 for human E and other cells. This may be due to differences in the availability of acceptor groups between the human and ovine E surface as the authors conclude, or due to the presence in human cells of regulatory proteins such as DAF. Alterations in the membrane concentrations of these regulatory factor could account for differences in experimental results and I have therefore studied the role of cell surface DAF numbers in determining SLE erythrocyteC4 isotype deposition (see results, chapter 5). Other work reported in this study is also at variance with the unitary hypothesis that C4 surface binding is solely determined by the availability of surface transacylation sites. The binding of C4A to cell surface glycophorin A via amide linkages was found to be relatively more efficient than C4B, despite the fact that hydroxyl moieties outnumber amino groups by several orders of magnitude in this molecule. The authors propose that these data may be explained by the existence of a secondary substrate binding site on nascent C4b.

1.24 Differential function of C4A and C4B *in vitro*

The role of the classical pathway in the physiological processing of immune complexes *in vivo* is discussed in chapter 1, section 5. However it is appropriate to refer to differences which have been demonstrated between C4A and C4B in the inhibition of immune precipitation (IIP, see section 1.51) and the complement/CR1 mediated adherence of immune complexes to erythrocytes at this point. Schifferli and colleagues have studied these functions of C4 isotypes *in vitro*. Initially [40], the relative IIP and haemolytic activities of C4A and C4B deficient sera were compared with serum from a donor known to posses 4 C4 alleles. The ratio of haemolytic activity of C4B/C4A (sheep E) was approximately 3:1, and C4B alone was more efficient in inducing haemolysis than the mixed isotypes in normal serum. IIP activity of the isotypes favoured C4A, with a ratio of 1.66, though in this case there was little obvious difference between C4A alone and mixed C4 isotypes in normal serum. This suggests that competition between isotypes when both are present may mask the subtle functional differences which exist. In this study it was noted that a quantity of serum containing C4 equivalent to 1% of the haemolytic activity of normal serum was sufficient to restore full IIP activity to C4 deficient serum. Concentration of C4 was the limiting factor since 1% normal serum on its own did not restore IIP activity. Paul and colleagues [41] have elucidated the reaction kinetics of IIP

using serum-free mixtures of C1 and C4 isotypes and a BSA-antiBSA complex similar to that employed by Schifferli. When the rates of IIP are compared, a greater difference in function was noted, with an average ratio of reaction rates of 2.8 between representative C4A3 and C4B1 containing sera. The steady state or maximal IIP observed showed a very similar increased efficiency of C4A compared with C4B to that observed by Schifferli. In comparing the efficiency of C4A and C4B in opsonising tetanus toxoid-anti-tetanus toxoid (TT/anti-TT) IC for binding to human erythrocyte CR1, Schifferli [42] concluded that C4A isotypes were 1.5-fold more efficient than C4B.

The preferential binding of C4A to amino groups could thus influence the relative efficiency of C4 isotypes in the processing of ICs of protein antigens in 2 main ways, either related to the efficiency of IIP reactions, or to the opsonisation of ICs for E-CR1 binding. Using purified, functional C4A and C4B, Gatenby [43] was able to compare the relative efficiency of these proteins in dynamic and steady state studies with both preformed and nascent immune complexes. While the difference in IIP function was slight in favour of C4A, there was a much more marked increase in the efficiency of C4A with regard to opsonisation for E-CR1 binding implying that alterations in C4A concentration *in vivo* may have disproportionate effects upon this facet of IC physiology.

The differential reactivity of C4 isotypes towards nucleophile substrate may also be of consequence in the pathogenesis of certain drug related lupus-like illnesses [44,45]. Sim and co-workers have hypothesised that the association of hydralazine and metabolites of procainamide and practolol with SLE-like syndromes may be due to the presence of nucleophilic residues in the drugs which are able to hydrolyse the C4 (and C3) thiolester bond, leading to potential reduction of C-related protective functions. In addition, D-penicillamine [D(-)-ßß-dimethylcysteine] (DPA), which can cause glomerulonephritis, showed preferential covalent binding to the activated thiolester of C4A, with concomitantly greater reduction in the haemolytic activity of C4A than C4B. While it is possible that the pathogenic (or therapeutic) effects of DPA are related to inhibition of C4 mediated functions, corroborative data such as linkage with C4A null alleles is missing.

The clearance kinetics of ^{125}I-C4 from plasma were studied [46]. The conventional view is a bi-exponential model, suggesting distribution through 2 "compartments". In contrast, these authors [46] found that a 3-exponential model gave the best computed fit to the experimental data, suggesting a 3rd compartment of distribution. The fractional catabolic and synthetic rates suggested by this model were 2.41+/-0.98%/hr and 0.165+/-0.089mg/kg/hr respectively. The ratio of distribution of C4 between extra and intravascular space predicted was 2.24, while the usually reported figure is 0.54.

1.25 Comparison of C4A and C4B

For reference, an overview of the major structural and functional differences between C4A and C4B is presented in table 1, below.

		C4A	C4B
1.	Electrophoretic mobility		
	(a) agarose gel	Fast (acidic)	Slow (basic)
	(b) SDS-PAGE (a chain)	Mr 96 000	Mr 94 000
2.	Thiolester/ relative covalent binding reactivity		
	(i) Amino groups	High	Lower
	(ii) Hydroxyl Group	lower	High
3.	Functional correlates (comparison with mixed A and B = 1)		
	(a) haemolytic activity	0.51	1.91
	(b) inhibition of immune precipitation	1.19	0.71
	(c) immune adherence (human erythrocytes)	1.24	0.81
4.	Typical antigenic determinants	Rodgers (Rg:1, 2)	Chido (Ch: 1-6)

Table 1: Comparison of the major properties of C4A and C4B

1.26 C4 isotype serological activity and molecular structure

A complex relationship exists between the molecular basis of C4 polymorphism and serological reactivity. Many studies, including work presented here depend on anti-C4 monoclonals to quantify C4 isotypes. The difference between the serological determination of C4 molecules and the molecular substitutions which confer isotypic identity is therefore of importance, since the equivalence between isotype and serotype is not absolute and this may lead to errors if not recognised.

Rodgers (Rg) and Chido (Ch) are human erythrocyte and plasma antigens originally defined by the alloantibodies anti-Rg [47] and anti-Ch [48], produced by individuals lacking the antigenic determinant after transfusion with Rg or Ch+ve blood. The genes encoding Rg and Ch were initially mapped to the HLA region by serological studies [49] and were subsequently localised to the α chain of the C4d fragment of C4 [50]. The identification of the Rodgers antigen as C4A and Chido as C4B was made by O'Neill [51]. These specificities are now known to result from the deposition on erythrocytes of small quantities of C4 from serum.

Methods based on inhibition of haemagglutination have been used extensively in the serological typing of the Rg and Ch antigens [52]. Plasma containing unknown C4 is incubated with anti-Rg/Ch of known specificity (or vice versa). The reaction mixture is then incubated with erythrocytes coated artificially with C4 from pool serum, and agglutination induced, after washing, by anti-human IgG. Recognition of antibody/antigen in the first reaction will reduce or abrogate binding to the erythrocyte/C4 reagent and will abolish agglutination. As an alternative approach erythrocytes may be coated with test C4 and the cell examined directly for its ability to be agglutinated by anti-Rg/Ch. If the anti-serum is completely absorbed in the first reaction, and no inhibition occurs, the plasma is referred to as a "complete inhibitor", failure of absorption and subsequent agglutination is termed a "negative inhibitor" plasma [52].

It was observed that certain plasmas produced only partial inhibition (pi) [47] which was not due to quantitative variation in C4. Further studies with specific C4 allotypes led to the conclusion that anti-Ch and anti-Rg were polyspecific and that there may be more than one antigenic determinant of Rg or Ch [53]. Giles [54,55] proposed a model of 2 Rg (Rg:1, 2) and 3 Ch (Ch:1, 2, 3) antigenic determinants and in all models Rg:1 and Ch:1 are the commonest. Further serological subdivisions of common allotypes were detected by the application of standardised polyspecific allo-antisera and comparison of this serologic data with isotypic characterisation soon led to the breakdown of the conventional division between C4A/Rg and C4B/Ch [56] with the recognition of a further 3 Ch determinants (Ch:3, 4, 6) [27]. These cumulative serological observations have enabled the following conclusions to be drawn.

1. Rg:1 and Ch:1 are alternative antigenic determinants but are not uniquely associated with C4A and C4B
2. Some C4A molecules may not express Rg:1 or 2 and may express Ch determinants but never Ch:2 and 4
3. All C4B molecules express Ch:4
4. Ch:2 and 5 are never split on the C4B molecule
5. Ch:3, 1 and 6 appear to be related on both C4A and C4B molecules

The molecular mechanisms underlying these serological relationships have been summarised [57]. C4A and C4B do not differ in post-translational structural modifications such as disulphide bond structure, degree of glycosylation and sulphation implying that inter-isotype differences are sequence related. The amino acid sequences derived from full length and partial cDNA clones of C4A4, C4A3 and C4B2 alleles, and limited genomic sequence of the C4B1a, C4B1b and C4B2 alleles [28-30] and other data [58] suggest there is <1% sequence variation (17 changes amongst 1725 amino acids) between C4A and C4B

and that 12 of these changes are clustered in a region of 140 amino acids approximately 230 residues C-terminal to the thiolester site.

Yu and colleagues [30] have compared sequence and serological data amongst 4 patients with informative C4 genotypes. The alleles C4A3a and C4B3 represent the "classical" serological findings, with C4A3a showing Rg:1, 2 and no Ch determinants, while C4B3 shows no Rg but all Ch reactivities. Alternatively the C4A1 and C4B5 alleles show reversed antigenicity: C4A1 is Rg -ve but expresses Ch:1, 3, 5 and 6 (not 2 and 4, as above), while C4B5 is Rg:1+ve and also expresses Ch:4 and 6. Sequence variations common to the two C4A proteins but not shared by the 2 C4B proteins must therefore be associated with isotypic, not serotypic determinants and vice-versa. Similarities between C4A3a and C4B5 allowed recognition of the Rg specific amino acids while comparisons amongst the C4B3, C4A1 and C4B5 sequences, plus cDNA and genomic sequences of other informative alleles have allowed conclusions to be drawn about the sequence determinants of Chido reactivities. On this basis the model illustrated in figure 4 (overleaf) was constructed. The main features of each of these informative haplotypes are shown in figure 5 (p32), a comparison of similar data relating to haplotypes helpful in the analysis of Chido specificities is shown in figure 6 (p33).

These serological differences were associated with 8 amino acid substitutions grouped in 4 regions as shown in figure 4. Regions I and III are single amino acid substitutions at positions 1054 and 1157 respectively, while the 4 changes comprising group II are found amongst the 6 bases between positions 1101-1106. The 2 changes comprising group IV are clustered in the 4 residues at positions 1188-1191. The only residues shared exclusively by both C4A and both C4B molecules are found in group II (1101-1106), which must therefore determine isotypic specificity (C4A molecules show PCPVLD, while C4B molecules show LSPVIH, variable amino acids are underlined). The C4B5, Rg+ve molecule shows the C4B isotype specific pattern in group II but acquires the same residues as the Rg expressing C4A3a molecule at region I (D) and group IV (VDLL). The opposite substitutions at group IV (ADLR) were seen in molecules expressing Ch:1. This reactivity is thus separate from the isotypic moieties and is exclusive of Rg:1. The C4B5 molecule studied here (results, chapter 3) expressed Ch:4 and 6.

Figure 4. Proposed structural model of the isotypic and serological determinants of human C4

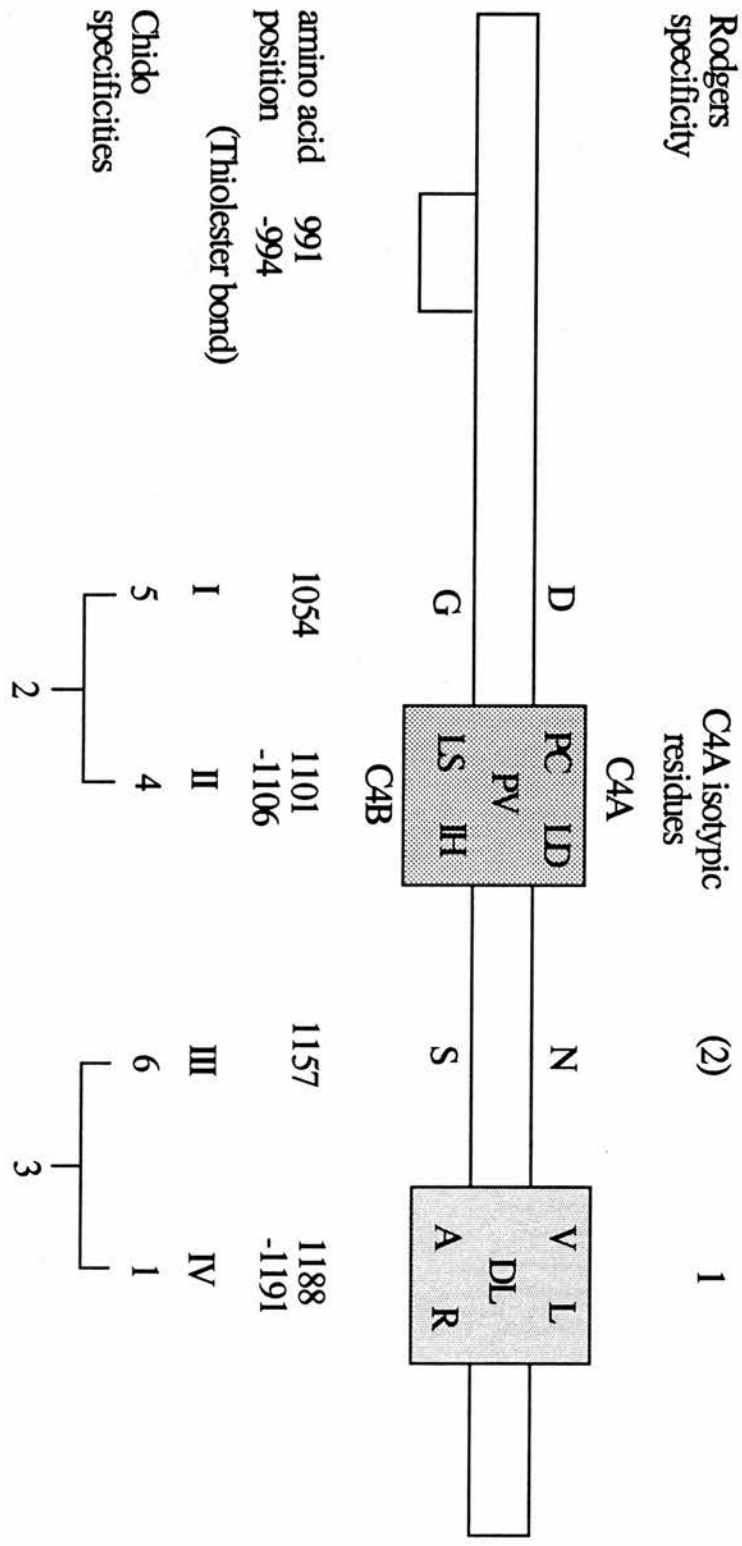

Figure 4. Proposed structural model of the isotypic and serological determinants of human C4. For legend see text.

Figure 5. Correlation of serological and structural data in 4 cloned C4 genes

Figure 5. Correlation of serological and structural data in 4 cloned C4 genes. For legend see text.

Figure 6. Comparison* of the Chido determinants and amino acid sequence of some C4 allotypes with a typical Chido positive allele (eg C4B3)

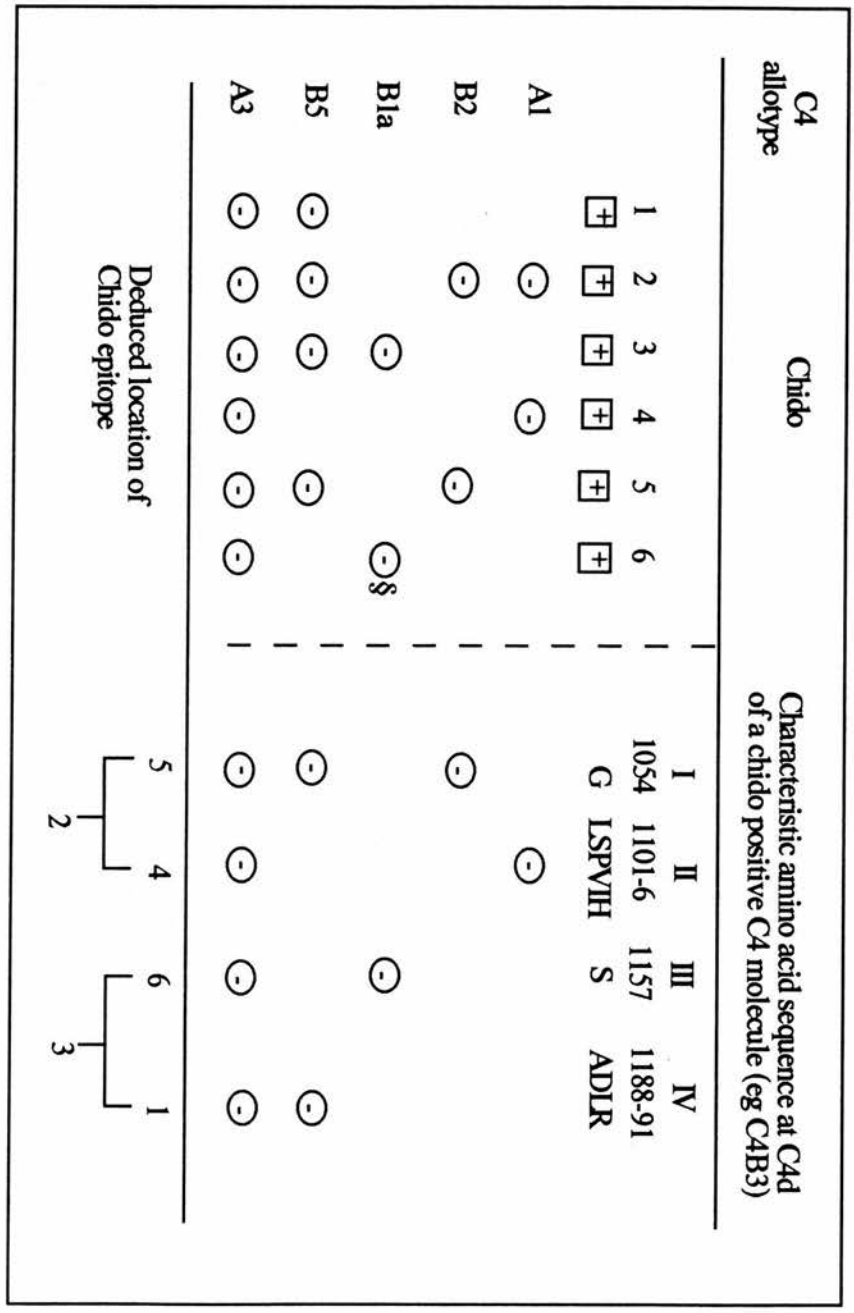

Figure 6. Comparison of the Chido determinants and amino acid sequence of some C4 allotypes with a typical Chido positive allele (eg C4B3). The negative symbol in the right hand panel denotes absence of the typical Chido amino acids. Substitutions are not shown.

By comparison with other C4B molecules it was concluded that the C4B isotype specific residues at group II specify Ch:4, which is therefore specific to C4B. In addition, the C4B5 isotype shares the S at region III (1157) with other Ch:6+ve C4B molecules, this substitution was therefore considered to specify Ch:6. The opposite (N) substitution may contribute to Rg:2 reactivity, though this is not certain. Finally, the G residue at region I (1054) is thought to determine Ch:5, while the reciprocal substitution seems to have no effect on Rg specificity though it does have other effects on Ch reactivities. These specificities appear to be determined by sequence variations alone, but there is not a unique relationship between amino acid substitutions and the Ch:2 and 3 determinants. For example, C4A1 differs from C4B3 only in the C4 isotype encoding region II, but loses both Ch:4 (as expected from the foregoing) and Ch:2, which is not explained on the basis of sequential data alone. A similar argument applies for Ch:3. It was therefore concluded that these are conformationally determined specificities and appear to be dependent on the correct apposition of Ch:5 and 4, and Ch:6 and 1 respectively. In practical terms, this model reemphasises that the serological recognition of Rg:1/Ch:1 is close to, but not identical with, the isotype of the molecule.

C4A and C4B isotypes are determined by the sequences PCPVLD and LSPVIH at positions 1101-1106 respectively. These amino acids therefore appear to determine many of the functional and physical differences between these 2 molecules including thiolester reactivity. The isotypic and thiolester regions are separated by 106 amino acids. Analysis of regional hydropathy suggests that the isotypic residues occupy a hydrophilic site which may be exposed in the tertiary structure of the molecule, while the thiolester bond is in a hydrophobic region, likely to be hidden in nascent C4. The conformational change which occurs on activation of C4 [59] contributes to activation of thiolester binding by exposing the thiolester site on the surface and may approximate the isotypic and thiolester sites. This would allow the interaction of residues at both sites and may explain the influence of isotypic residues on the binding reactivity of the molecule. The negatively charged Asp at 1106 in C4A may increase the nucleophilicity of nearby amino/amine groups towards the thiolester bond and thus lead to the observed preferential reactivity of these groups with C4A [37]. Alternatively, the positively charged HIS 1106 of C4B may participate in hydrogen-bonding charge shift in hydroxyl substrates resulting in more the efficient binding of C4B to hydroxyl groups of carbohydrate antigens. The activation conformational shift may thus both expose the thiolester for reactivity and contribute towards creating an environment which may be catalytic for the nucleophilic attack of preferred amino/hydroxyl groups upon the thiolester carbonyl.

Certain other molecules may contribute towards isotypic differences, for example, Pro 1101 in C4A (along with Cys 1102 and the shared Pro 1103) may induce conformational constraints on the molecule and result in the slower electrophoretic mobility of the C4A α chain found in modified SDS-PAGE gel electrophoresis [57]. It is of interest that the mouse has one functional C4 gene and shows a hybrid structure *i.e.*PCPVIH. Dieli and colleagues [60] have noted that C4 from BALB/c and CBA/J differ in their capacity to inhibit the transfer of delayed hypersensitivity by murine lymph node cells. Pretreatment of the C4 with glycine and glucose produced opposite effects on the C4 function of each species arguing that the binding reactivity of the thiolester moiety in each of these mouse strains favour amide or ester bond formation unequally. The amino acid sequences of the C4 isotypic sites in these strains has not been ascertained.

1.27 The macromolecular arrangement of the C4 loci: polymorphism of C4 gene number and size

A feature of the C4 locus is the high frequency of silent, null or non-expressed alleles (designated quantity 0, C4Q0) at either locus. A variety of cDNA genomic probes and specific restriction enzyme cleavage sites have allowed RFLP analysis of variation in gene size, the macromolecular arrangement of alleles within the C4 locus (see figure 3) and the structural basis of genetic deletions leading to common C4 null alleles (figure 7, overleaf).

Taq 1 digestion of genomic DNA separates the C4 loci and their associated steroid 21-OH genes by restriction of a site near the 5′ end of the molecule, but is not affected by the nature of the C4 gene encoded. Using this enzyme, Carroll and others [61,62] have shown that the C4Q0 found in a large proportion of haplotypes is due to deletion of a C4 gene. The defect in some non deleted haplotypes was elucidated by Yu and Campbell [63] who used a C4d specific, 927kb Bam H1 probe in Southern blot analysis of restriction fragments after digestion with N1a IV, EcoO 109 and Taq 1. Isotypic differences are associated with 2 N1a IV fragments for C4A (276 and 191bp respectively), while restriction of a C4B gene results in a single 467bp fragment. EcoO 109 separates C4 molecules by recognition of site related to the Rodgers and Chido epitopes, rather than isotype. Thus a Rg1 bearing C4 gene is represented by a 565bp EcoO 109 fragment, while a Ch:1 bearing C4 gene shows a 458bp fragment with this enzyme. Comparison of the RFLP produced by these enzymes with the taq 1 polymorphism demonstrated that the C4B null allele in the haplotype B44, DR6, C4A3, C4BQ0 was associated with the presence of a homoduplicated C4A gene in the C4B locus. In this thesis I will present data which suggest that this homoduplicated C4A gene may be expressed. No non-expressed or pseudo-C4B alleles have been detected [63].

Figure 7. Molecular map of common deletions in the C4 locus producing null alleles

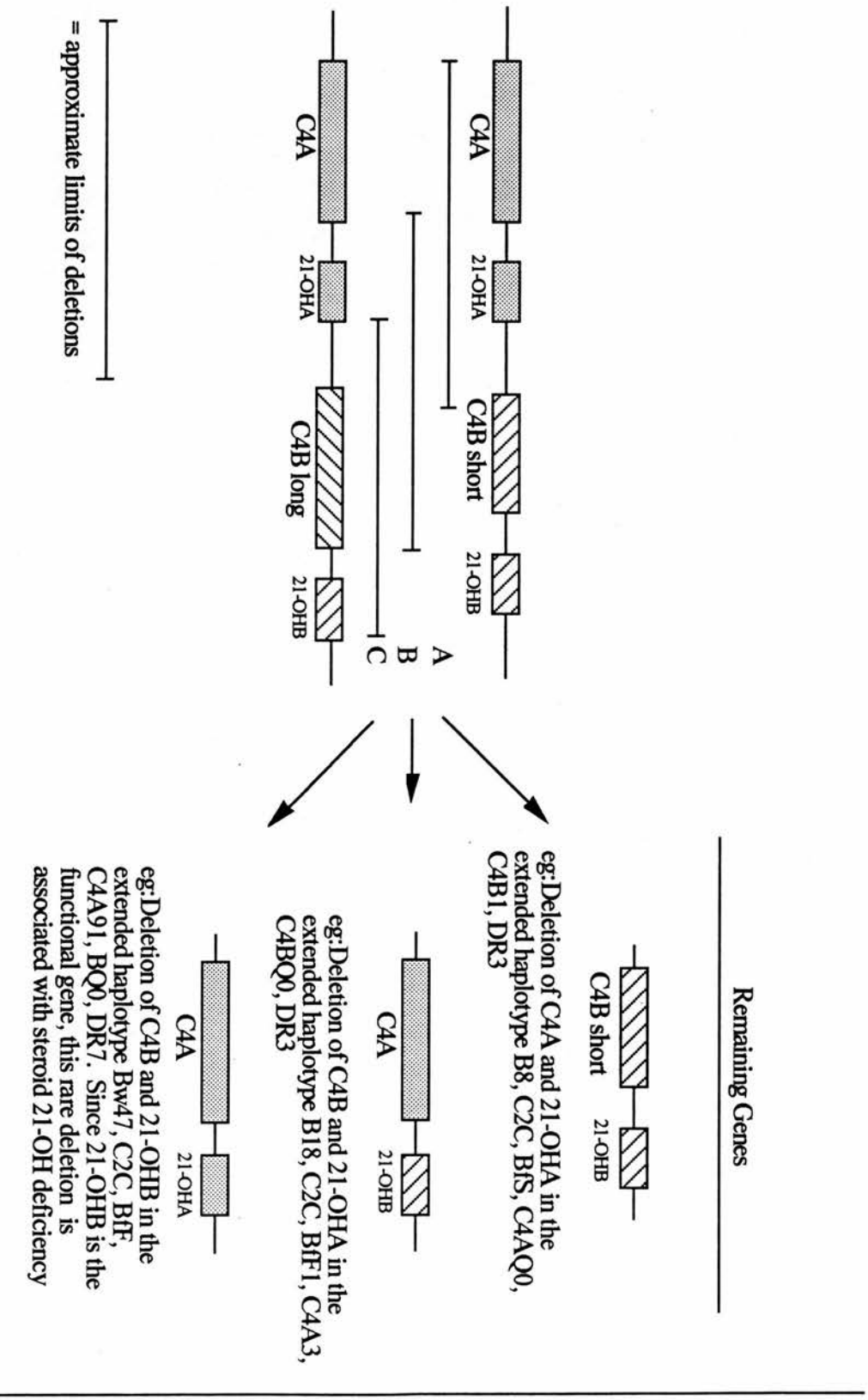

Figure 7. Molecular map of common deletions in the C4 locus producing null alleles. Redrawn from Hauptmann et al [80]

The macromolecular organisation of the C4/steroid 21-OH complex, and the detailed intron/exon structure of the C4 genes have been studied in detail [30,62,63]. The two C4 genes are separated by 10kb, and each has a cytochrome-p450 steroid 21-OH gene within 2kb of the 3′ end. The 21-OHA and 21-OHB genes are each 3.5kb and show an overall sequence homology of 98%, but only the 21-OHB gene is transcriptionally active. Taq 1 RFLP analysis divides the C4 locus into locus I usually encoding a C4A gene and 21-OH A gene, while locus II usually encodes C4B and the 21-OH B gene. Locus I is characterised by a 7.0kb 5′ fragment, while locus II shows a 6.0 or 5.4kb fragment. The size of these genes differs with the presence of a 6.5kb intron 2-2.5kb 3′ to the transcription start site at the 5′ end of the gene. C4 genes may therefore be long (approx 22kb) or short (approx 16kb). To date both long and short C4B genes have been identified but no short C4A genes have been found. Otherwise the detailed intron/exon structure of the C4 locus seems to be stable between both loci [30].

1.28 The regulation of C4 gene expression

Little is known directly about the genetic mechanisms which regulate C4 expression in humans, though information relating to murine C4 and Slp (sex-linked protein, the non-functional murine C4 analogue) is available. Observations relating to the role of C4 in the murine acute phase response and to the relationship between C4 genotype and phenotype are also of relevance.

Slp and C4 are homologous proteins encoded by duplicated genes in the S region of the murine H-2 (major histocompatability complex). Murine C4 levels are primarily determined by S region genes for high and low expression. Slp may be present (Slpa), or genetically deficient (Slp0). Slpa expression is influenced by an androgen sensitive controller situated in the 5′ flanking region [64], though a variety of other regulatory influences have also been reported [65]. Pre-translational, Non-H-2 (trans-regulatory) [65], tissue specific [66] effects were found on the expression of both murine hepatic C4 and Slp. These data point to the complexity of regulation in the murine C4 and Slp genes where both cis and trans-regulatory mechanisms are apparent and may be due to the incorporation of a retroviral controller sequence [67]. No equivalent data is available in humans, but the observations of Tuedsson and colleagues [68] that there is considerable variability in the expression of C4 isotypes in subjects with the same MHC extended haplotypes, may suggest similar multiple regulatory mechanisms. However, variation in C4 turnover rates would have to be established before the evidence for this was considered exhaustive.

While the majority of C4 is synthesised in the liver in humans, there is recent evidence of tissue specific regulation of C4 synthesis in the kidney. Deposition of classical pathway components in renal tissue is usually considered evidence of immunopathology. However, Feucht and colleagues detected both C4A and C4B in the mesangium of normal renal glomeruli [69]. More recently, members of the same group have reported the finding of C4 messenger RNA in renal interstitial cells at levels 25% of those found in liver, suggesting that in-situ production of C4 may be related to normal renal (mesangial?) function [70]

In the mouse MRL/lpr model of SLE, intraperitoneal administration of inflammatory stimuli induced differing effects on the expression of C4 and Bf [71]. Murine C4 production in response to *C. parvum.* and *C. bovis.* was found to be dependent on the consitutive genetic level of C4 production [72]. No data is available on the acute phase expression of C4A and C4B in humans *in vivo*, but the behaviour of human C4A and C4B genes has been studied in a mouse fibroblast transfectant model [73]. In this system, expression of C4 genes was not affected by interleukin-1 or tumour necrosis factor, while interferon alpha induced a greater increased in the expression of C4A than C4B, suggesting potential heterogeneity in the acute phase responses of C4 isotypes. An attempt was made to investigate differential acute phase responses of C4 isotypes in humans and is reported in results: chapter 3.

Section 3
Complement Deficiency and Inherited Heterozygous Deficiency of C4A in SLE

Introduction

Homozygous deficiency of classical pathway proteins are amongst the most powerful genetic factors known to contribute to disease susceptibility in SLE. Complete deficiency of C4 is virtually sufficient in itself to induce disease and most individuals described with this deficiency have SLE or a similar illness. While homozygous C4 deficiency is rare, heterozygous deficiency at the C4A locus is common and is also associated with SLE. The theory has been advanced that this association is the result of reduced efficiency of immune effector mechanisms (complement mediated IC clearance) which allows persistence of antigen and prolonged stimulation of the immune response [1]. In this chapter, I will discuss evidence for the association between inherited deficiency of complement and SLE with special reference to heterozygous C4A deficiency.

1.31 Complement deficiency and disease

Genetic deficiencies of nearly all of the complement components have been described and the subject has been extensively reviewed [74-76]. The arguments linking effector mechanisms and SLE have recently been summarised [1]. Homozygous deficiency of complement is associated with 2 main sets of clinical problems, infection and immune complex disease. The relative incidence of these problems in described complement deficiency states is summarised in table 2 (overleaf, redrawn from [1]). This data is likely to be incomplete and may be influenced by selection bias of various types It may therefore not represent an absolutely rigorous treatment of the overall problem of the incidence and disease associations of C deficiency [1]. However, the overall pattern of disease associations is unlikely to be altered by addition of further information and, specifically, no homozygous C4 deficiency has been ascertained in the healthy populations screened to date. The preponderance of Neisserial infections in deficiency of C3 and the later pathway components is explained by the relative protection from specific immunity conferred by the intracellular localisation of these bacteria and thus the greater requirement for fluid phase, alternative pathway mediated bacterial lysis.

Component	Number with deficiency	Numbers with associated diseases	
		IC disease	Infection
Classical pathway			
C1q	15	14	many with pyogenic infections
C1r or C1s	8	6	"
C4	16	14	"
C2	66	38	few with pyogenic infections
C1 inhibitor	> 500	2-5%	"
C3 and alternative pathway			
C3	11	8	10 pyogenic (mainly Neisseria)
B	0	—	—
D	2	2	pyogenic
Properdin	3	—	2 Neisseria
(3 more may have died of fulminant infection prior to ascertainment)			
I	5	1	4 pyogenic
H	2	1	(haemolytic uraemic syndrome)
Membrane Attack Complex			
C5	12	1	9 Neisseria
C6	17	2	10 Neisseria
C7	14	1	6 Neisseria
C8	14	1	8 Neisseria
C9	many		no disease association

Table 2. Reported cases of complement deficiencies and associated diseases. From [1]

1.32 Null alleles of C4 in SLE: inheritance and molecular genetics

Homozygous deficiency of C4 is a rare condition, especially given the frequency of partial deficient C4 haplotypes [76]. The C4A and C4B loci are closely linked to each other and segregate together in marked linkage disequilibrium as C4 haplotypes. Moreover, the 4 complement genes of the class III region C2, Bf C4A and C4B are inherited together as single genetic units in man, called "complotypes" [77] which also show pronounced linkage disequilibrium. Complotypes are described in the order written above which reflects the known chromosomal arrangement of the genes. Complotypes themselves show a further level of linkage disequilibrium and are inherited in association with extensive regions of MHC DNA in "extended haplotypes" [22] sometimes referred to as "supratypes"

[78]. The degree of polymorphism within the C4 loci has been described in chapter1, section 2. An unusual feature of C4 polymorphism is the high frequency of null alleles, which have been found in all populations studied (figure 8: overleaf). From the frequency of C4 null alleles, the proportion of individuals with 4, 3, 2, and 1 alleles may be estimated (shown in figure 8). From these estimates it will be seen that only 50-60% of the normal population posses 4, 30-38% posses 3 and 5-10% posses 2 C4 alleles, making heterozygous deficiency the commonest immunogenetic abnormality described [80]. C4Q0 are also inherited in strong linkage disequilibrium and table 3 (below) shows some of the commoner extended haplotypes encoding this deficiency.

HLA			Complotype				HLA	Frequency
A	Cw	B	C2	Bf	C4A	C4B	DR	
1	7	8	C	S	0 (del)	1	3	0.0815
2/11 [A]	5	44	C	S	3	0 (no del)	4	0.0636
3	4	35	C	F	3, 2[b]	0 (no del)	1	0.0359
30	5	18	C	F1	3	0 (del)	3	0.0191
23/29 [A]	4	44	C	F	0	1	7	0.0089
2	3	W60	C	S	0	2	w6	0.0065

Table 3: The commonest extended haplotypes encoding a C4A or C4B null allele
[A] alternative alleles, del/no del = deleted or non deleted
[b] duplication of the C4A allele or gene conversion B to A

The haplotype A1, B8, DR3, BfS, C21, encodes C4AQ0, C4B1 and is the commonest of all caucasoids haplotypes (the extent of this molecular deletion is shown in figure 3). Most C4B null alleles are also found in a small number of specific extended haplotypes. It might be expected that crossover events amongst common single null haplotypes would lead to existence of a number of double null haplotypes. However, the double null haplotype is exceptionally rare [1] and does not occur within specific extended haplotypes [79]. The molecular basis of the C4A deficiency in the cases examined by Uring-lambert was different from the gene deletion found in the common A1, B8, DR3 haplotype (see below) and resulted from parental consanguinity. Moreover, the genetic mechanisms leading to homozygous deficiency seem to involve crossover between individually rare haplotypes more frequently than expected. The conclusion is that the genetic events leading to the occurence of homozygous deficient C4 haplotypes seem unique to each individual.

Figure 8: Estimates of the proportion of individuals with 4, 3, 2, 1 and 0 functional C4 genes in the normal Caucasoid, Negroid and Mongoloid populations.

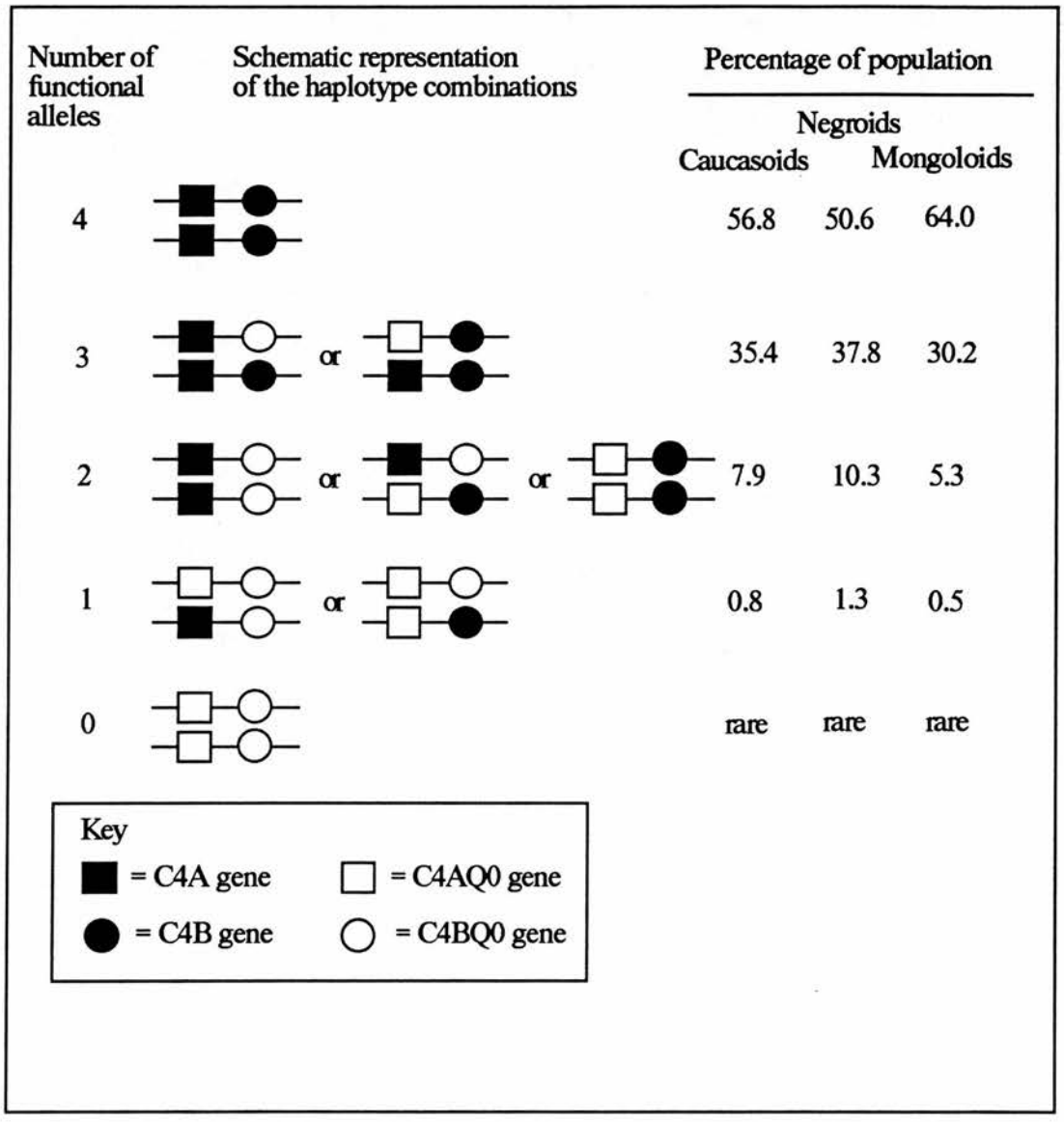

Figure 8: Gene frequencies of C4 deletions calculated from the Hardy-Weinberg expectations with the frequency of C4A and C4B alleles established during the IXth International Histocompatibility Workshop. (redrawn from table 3 in Hauptmann et al 1988, [80])

By contrast to the individually unique mechanisms seen in homozygous C4 deficiency, a relatively restricted number of genetic mechanisms seem to explain the majority of common C4 null bearing haplotypes.

These include:

1) the presence of a defective or mutant C4 gene
2) conversion of a C4B gene to a C4A gene.
   This can lead to two different patterns of gene expression:
   (i) 2 identical allotypes at each locus (homo-expression, homo-duplication)
   (ii) 2 alleles of the same isotype but differing allotype eg A3, A2, BQ0 (iso-expression)
3) gene deletion: the extent and distribution of common gene deletions are shown in figure 7

DNA analysis has shown that about 50% of common null alleles are due to deletion of a 28kb fragment involving either the 21-OHA or 21-OHB genes and 3 common patterns have been found [61,62]. These include (A) the C4A and 21-OHA genes, (B) the C4B and 21-OHA or (C) the C4B and 21-OHB genes, but not both C4A and C4B genes together (figure 7).

1.33 Homozygous C4 deficiency and immune complex disease

The clinical findings associated with complete C4 deficiency have recently been reviewed [80]. The majority of patients have presented with SLE or, in the absence of full diagnostic criteria, an SLE-like illness. These patients have been characterised by early onset (majority <10yrs), prominent skin lesions (or Discoid LE) and Raynaud's phenomenon but mild renal disease. Serologically, antinuclear antibodies are seen in 58%, but anti-dsDNA have not been described. Interestingly, 5 of 12 patients possessed Ro but no patients had La antibodies, a pattern similar to that seen in C2 deficient patients. The association of C4AQ0 with SLE has been documented in at least five series of studies (see Hauptmann [76] for review) and homozygous deficiency was found in an average of 11.7% of patients compared with 0.9% of normal controls.

Recently, a Canadian family have been reported in whom C4 deficiency and Ro antibody production appear to segregate separately [81]. This emphasises the finding from other familial cases that multiple influences, including genetic loci outwith chromosome 6 are involved in the development of susceptibility to SLE in the majority of cases. A further family have been studied in whom almost complete deficiency (2-5% normal levels) of C4 was inherited as a non-HLA linked autosomal dominant gene [82].

1.34 Heterozygous C4A deficiency and SLE

An association of C4QO encoding extended haploypes with several autoimmune diseases, including Graves disease and insulin dependent diabetes mellitus as well as connective tissue diseases such as Sjogren's syndrome, Henoch-Schonlein purpura and SLE has been noted [76,78,83]. Heterozygous deficiency of C4 alleles, specifically C4A, is associated with SLE. The family study of Fielder et al [84] was the first to report the presence of null alleles and the associated extended HLA haplotype of a group of SLE patients and their families. Null alleles (of C4A, C4B or C2) were found in 83% of patients and in 43% of controls. The majority of deficiencies in patients were heterozygous C4AQ0 (78% of null alleles). 82% of C4 nulls were present on extended haplotypes which also encoded DR3. Of these, the A1,B8, DR3 haplotype was the most frequent and was present in 15/22 C4AQO haplotypes in patients. The authors concluded that linkage disequilibrium made it impossible to tell whether the C4 null allele, DR3 or another, linked gene was most important in determining the disease association, though Green et al [85] have reanalysed the data by the empirical logistic method and concluded that the association is strongest with the C4A null allele.

Evidence to suggest that it may be the C4A null allele which is the important gene comes from genetic studies in other populations,where C4Q0 also show association with SLE but are encoded with different HLA alleles (extended haplotypes). In the Japanese, DR3 is a rare allele and DR2 was found to be the most common HLA antigen in patients with SLE [86,87]. Similarly, in a Chinese population C4A null and DR2 were statistically associated with SLE, while C4BQ0 alleles were not [88]. In Black American SLE patients the most powerful association was with DR3 [89]. Howard and colleagues [90] have demonstrated an independent contribution of risk from C4AQ0 genes and DR2 in a study of Caucasoid and Black American patients with SLE. In this population, C4AQ0 linked B8 and DR3 genes did not contribute a separate risk factor, while in Green's analysis of Fielder's data there did appear to be an additive risk from the presence of both C4AQ0 and DR3. So and colleagues have confirmed a small increase of DR 2 in British Caucasoid patients with SLE [91]. This was unrelated to the predominant association of disease with the C4AQ0 bearing A1, B8, DR3 haplotype. Despite differences in HLA linked genes, calculations of relative risk of C4AQ0 for SLE from the study of Fielder and from a Japanese population (quoted by Lachmann and Walport [1]) demonstrated an association between C4AQ0 and SLE in both ethnic groups. The presence of C4AQ0 has a relative risk of 8.2 in Caucasoid SLE and 4.3 in the Japanese population, while C4BQ0 showed relative risks of 0.65 and 0.13 in these populations respectively. The molecular basis of C4A deficiency in SLE is heterogeneous [92,93], though most cases are due to the C4A steroid 21-OH deletion typical of the Caucasoid A1, B8, DR3 haplotype.

1.35 Acquired Complement deficiency and immune complex disease

A second line of evidence linking functional deficiency of classical pathway components and disease expression in SLE comes from the occurance of IC disease in individuals with acquired C deficiency. Heterozygous C1 esterase inhibitor deficiency is one of the commonest genetic C deficiency states and leads to an acquired reduction of C4 and C2 due to excessive C1-mediated turnover secondary to depletion of C1-INH at times of physiological stress [74]. In addition to hereditary angioneurotic oedema, approximately 2-5% also have an illness which is described as similar to SLE. The majority have LE-like skin disease, with photosensitvity and may have a positive lupus-band test [94]. Anti-nuclear antibodies are usual and mild abnormalities of urinary sediment are common. More severe proliferative glomerulonephritis has also been reported (see [94]). The locus controlling C1-INH production is not liked to the HLA, excluding genetic linkage as a cause for this association and suggesting that it is the loss of C function which is important.

1.36 Correlation of C4 genotype with phenotype and the measurement of C4 isotypes

The relationship between phenotype and genotype in subjects with heterozygous C4A deficiency has been difficult to ascertain exactly. It would be predicted that absence of 1 allele would lead to reduction of 25% of total C4, and 50% of C4A. The ratio of C4A to C4B in the presence of 1 C4AQ0 should be 0.5. When either 2 or 1 copy of each allele is present the ratio will be 1, while a single null C4B will produce a ratio of 2. The use of densitometric scanning of electrophoretic gels and confidence intervals for observed C4A/C4B ratios may thus help in the identification C4 genotype from phenotypic information [95,96], but are prone to error in a number of ways. Firstly, the presence of both C4A and C4B nulls cannot be differentiated from the presence of 4 alleles. Secondly, an expressed homoduplicated C4A allele is likely be incorrectly assigned, probably as a B null gene. More direct measurement by means of an ELISA assay utilising monoclonal anti C4A and C4B antibodies has been obtained [97]. While the ranges of serum C4A or C4B concentration show broad correlation with C4 gene numbers the range of serum values associated with each genotype is very wide and phenotypic overlap is extensive. There is also evidence that different individuals with identical extended haplotypes show marked variation in C4 phenotype [68]. These factors, and the problems related to the serological recognition of C4 isotypes (chapter 1,section 2) make the relationship between phenotype and genotype inexact even in normal populations. In patients with SLE, who are likely to be subject to variable rates of C4 turnover and who are generally hypocomplementaemic, the relationship is likely to be further obscured. However, there are reports of an identifiable effect of C4Q0 on total C4 levels in SLE. Wilson and colleagues [98] did find

a reduction in mean total C4 in SLE patients and controls with known *homozygous* deficiency of C4A or C4B, though in one C4B deficient patient, C4A levels were supranormal [99]. In a further study by this group [100], an association was found between disease activity, the presence of aCL antibodies and hypocomplementaemia in patients who had a single null allele, as estimated by plasma densitometry. During periods of disease quiescence no differences between serum C4 in patients with or without C4 null alleles, or associated with aCL antibodies were found. However, the difficulties in accurately determining the initial genotype from phenotype in these patients must be borne in mind, and to date there is no study in which C4 isotypes have been measured in a group of SLE patients who have been accurately genotyped by family study.

Section 4

Erythrocyte Complement Receptor Type 1 and other Complement Receptors

Introduction

Complement activation is one of the earliest events in host immune responses to foreign antigens. Coating of antigen, or immune complex, with complement opsonises it for interaction with immunocompetent cells and also, in primates, for processing by erythrocyte CR1. These effector functions are mediated, on a variety of immune and other cells, by surface receptors for complement fragments.

Four major receptors have been characterised and are primarily receptors for the activation and catabolic fragments of C3 and C4. A number of other cell surface proteins such as DAF and C4bp (reviewed in [101]) also have complement binding activity and function principally as regulatory factors in the complement cascade. It is a feature of these receptors that ligand binding preferences overlap extensively. Functional specificity is determined partly by the differences which do exist, and partly by the function of the receptor bearing cell. Table 4 (overleaf) outlines the major characteristics and cellular distribution of these complement receptors. All can bind iC3b, emphasising the overlap in ligand binding amongst these major CRs. Therefore, given adequate ligand concentration, a neutrophil could bind via CR1, CR3 and CR4 simultaneously. Similarly, lymphocytes may bind iC3b via CR1 and/or CR2 (B and some T cells) or via CR3 (NK cells). However, there is evidence that differing ligand affinity may result in quantitative modulation of binding. For example, with sheep E bearing 10,000 iC3b molecules, rosetting with neutrophils is mediated by CR3. In the presence of 10,000-40,000 iC3B/E, rosetting via both CR1 and CR3 occurs, while >45,000 iC3b molecules are required to recruit CR4 in these rosetting reactions [101].

1.41 Erythrocyte complement receptor type 1

Erythrocyte CR1 is the principal receptor involved in the physiological disposal of CIC in primates. I will therefore describe this molecule in detail and give an outline of the other major complement receptors (CRs).

The biology of CR1 has been the subject of recent reviews [102,103] Research leading to current understanding of this polymorphic, glycoprotein cell surface receptor has followed three phases.

Table 4. Membrane receptors for bound fragments of C3 and C4

Receptor	Specificity	Structure/Mr	Cellular Distribution					
			erythrocytes	platelets	granulocytes	macrophage/ monocytes	lymphocytes	other
CR1	C3b>C4b>iC3b C3i C3c	Four allotypes 160, 190, 220 and 240K	√	√ (non-primate)	√	√	√ B and some T	eosinophils mast cells dendritic cells glomerular podocytes
CR2	iC3b = C3dg >C3d »C3b Epstein Barr virus	145 K					√ B cells	dendritic cells
CR3	iC3b *S. epidermidis* *S. cervisiae*	Heterodimer 165 k **α** chain 95K **β** chain			√	√		NK cells dendritic cells
CR4 (p150, 95)	iC3dg, iC3b >C3d	Heterodimer 150 k **α** chain 95K **β** chain		√	√	√		
gp45-70 membrane co-factor protein(MCP)	C3b, iC3b and C4b (to the C3c region)	45-70K			√	√	√ B and T cells	
DAF decay accelerating factor	C3b, C4b	70K "sticky foot molecule"	√	√	√	√	√ B and T cells	endothelial cells

Table 4.. Membrane receptors for bound fragments of C3 and C4. For legend see text.

Early observations of complement dependent cellular interaction with micro-organisms were followed by formal description of the phenomenon of immune adherence (IA) and finally by detailed understanding of the molecular biology and functions of CR1. The binding of microorganisms to platelets was described by Bull in 1915 [104]. The involvement of antibody and complement in these reactions was inferred by Rieckenberg, Kritschewsky and Tscherikower (reviewed by Walport [102]). In 1930, Duke and Wallace [105] associated similar binding reactivity with primate erythrocytes and in 1938, Brown and Broom described polymorphism of human erythrocyte binding with opsonised trypanosomes [106]. The term "immune adherence" was used by Nelson in 1953 when he re-described antibody dependent binding of treponemes and pneumococci to human erythrocytes, along with the observation that these microorganisms were also opsonised for phagocytosis [107].

Recently, Fearon and his co-workers have contributed to the detailed biochemical and molecular biological characterisation of the receptor responsible for IA reactions. A glycoprotein of Mr 205,000 (gp 205) was initially released from erythrocyte cell membranes by treatment with detergent (Nonidet P40) and subsequently purified by extensive chromatographic procedures including affinity separation on sepharose-C3 [108]. This protein was inhibitory in the alternative pathway of complement activation as a result of its ability to displace Bb from C3b in C3bBbP, the properdin (P) stabilised alternate pathway C3 convertase. Additional actions in the catabolism of C3 were noted and CR1 is now known to be an alternative co-factor to H in the initial Factor I-mediated hydrolytic cleavage of C3b to iC3b, and to be the sole cofactor to I in the subsequent hydrolytic degradation to C3c (released) and cell bound C3dg [109-111]. Fearon also demonstrated that the C3b receptor function of human erythrocytes, polymorphonuclear leucocytes (PMN), B lymphocytes and monocytes was inhibitable by the F(ab´)2 fraction of monospecific murine anti-gp205 [112]. CR1 is also known to be present on the surface of glomerular podocytes [113] and on follicular dendritic cells [114]. Receptor numbers differ amongst these cell types. Fearon's calculations based on these studies with anti-gp205 (a polyclonal antibody) suggest mean numbers of 950 CR1/E, 21,000 sites/B cell, 57,000/PMN and 48,000/monocyte.

1.42 CR1 ligand binding

CR1 Ligand binding may involve both hydrophobic and charge interactions [108]. The binding site of CR1 to C3 (and C4) appears to be in the C3c (C4c) region [115,116] and CR1 binding is lost when iC3b (iC4b) is degraded to C3dg (C4d) by the liberation of the c fragment. C3b is the principal CR1 ligand, and affinity falls in the series iC3b, C4b and

iC4b. CR1 binding to C3b requires the availability of at least divalent ligand sites [117] and is not inhibited by the presence of monomeric fluid phase C3b. CR1 binding is most efficient with clustered ligands and rosetting is dependent on the density of C3b deposition (reviewed in [103]). Moreover, when C3b is deposited by the classical pathway, CR1 dependent rosetting is increased by the proximity of C4b, suggesting that an important function of low affinity C4b binding may be to enhance CR1 binding to C activating immune complexes. Fixed iC3b is a functionally important CR1 ligand and the importance of clustering and co-operation with C4b seems to be greater when the lower affinity interaction between this ligand-receptor pair is considered [118].

1.43 Molecular biology of CR1

(A) Structure

The majority of the coding sequence of CR1 has now been mapped by overlapping cDNA clones [119,120]. A model of CR1 structure has been constructed [101] and is characterised by the presence of 4 repeated sections of 450 amino acids sharing 70-95% identity and described as long homologous repeats (LHRs). Each LHR is comprised of 7 repeated sequences of 60-65 amino acids, termed short consensus repeats (SCRs). Each SCR possesses a highly conserved core of 10-15 amino acids which is also found in other C3 and C4 binding proteins and probably represents common genetic ancestry. The genes encoding a group of C3 binding proteins (CR1,CR2, C4bp, DAF and Factor H) have been mapped to band q32 of chromosome 1 and are described as the regulation of complement activation (RCA) locus [121]. The genes encoding CR1, CR2, DAF and C4bp are closely associated on an 800kb section of DNA within the RCA, while Factor H is separated from these genes by an unknown stretch of DNA [121]. The conserved SCR motif is also found in other C3/C4 binding proteins such as Factor B and C2, and in some which do not have complement binding activity such as IL-2 receptor and ß2-glycoprotein I.

(B) CR1 molecular polymorphism:

CR1 shows 2 molecular polymorphisms, one structural and one (on erythrocytes) numerical. The commonest structural alleles, designated F and S by Wong and colleagues [122,123] are found in 70% and 3% of individuals and encode proteins of 190Kd and 220Kd, respectively. Heterozygotes accounted for 27% of those studied and show the interesting phenomenon that the proportion of allele expressed by the erythrocytes of different individuals is markedly variable, while leucocytes express greater quantities of the higher molecular weight species. The basis of this polymorphism seems to be differences in the numbers of LHR regions encoded by the allele [123], and at least two other minor variants of 160Kd and 250Kd have also been recorded [124]

Differing cell types express markedly different numbers of CR1 (see above). Whereas expression on leucocytes does not vary significantly between individuals, ECR1 numbers may vary as much as 10-fold due to polymorphism in the genes controlling ECR expression. The early observations of Brown and Broom may be taken to suggest the existence of ECR1 numerical polymorphism in that E drawn from Brown consistently failed to mediate immune adherence reactions with opsonised trypanosomes. Using Hind III digestion and a 0.75cDNA CR1 probe, the presence of 2 RFLP alleles corresponding with numerical expression has been demonstrated [125]. All individuals studied possessed one or both of these RFLP bands, consistent with allelic inheritance. Homozygous expression of a 7.4kb band was associated with high CR1 numbers (611+/-33, mean and SEM), while the rarer homozygous 6.9kb phenotype was associated with lower receptor numbers (156+/-13). Mean receptor numbers associated with the heterozygous state were 455+/-52 and overlapped extensively with the high, but not low, receptor number groups. Correlation of cDNA probe mapping to the q32 band of chromosome 1 with structural alleles in family studies demonstrated that the regulatory region is associated with the structural gene, is cis-acting and is likely to be a transcription enhancing element [125]. Furthermore, other factors can modulate the regulatory effect of the 7.4kb high expression allele on receptor numbers. Evidence for this includes the observation that 7.4kb homozygotes show a wide spread of receptor phenotype, while 6.9kb homozygotes do not, and also that low expression of structural alleles associated with the 7.4kb regulatory element is seen in certain individuals. The action of these proposed regulatory element is tissue specific, in that B Cells and neutrophils showed no phenotypic differences associated with their inheritance.

1.44 CR1 function

The role of ECR1 in immune complex handling is discussed in chapter 1, section 5, while I will here consider other ECR1 functions and CR1 ligand binding in other cell types.

Erythrocyte CR1 function

The co-factor activity of CR1 in the degradation of surface bound C3 (and C4) has been discussed. Unlike DAF [8], the unusual structure of CR1 may allow it to extend its C3 catabolic role beyond the confines of the erythrocyte (or other cell) surface and thereby to protect nearby cells or biological surfaces from the phlogistic effects of complement deposition. Fixed iC3b is able to interact with receptors of a variety of other immunologically competent or phagocytic cells (Table 4) including neutrophils, monocytes, natural killer cells, follicular dendritic cells and platelets. Degradation to C3d restricts cellular interaction to cells bearing CR2 (B lymphocytes), thus reducing or altering the potential biological sequelae.

Phagocytic cell CR1 function

In broad terms, the presence of CR1 on phagocytic cells allows binding, endocytosis and phagocytosis of opsonised particles, though the relationship between receptor binding and cellular activation in the mediation of these functions is complex. Neutrophils are able to bind and ingest opsonised particles via CR1, but it probably requires interaction of the particle with other receptors such a IgG Fc receptors (FcR) before a respiratory burst can occur. Alternatively, activated monocytes and macrophages will phagocytose particles after CR1 ligand binding alone.

The CR1 of unstimulated phagocytes resides in two pools; 20% (approximately 3000 CR1/cell) is expressed in clusters on the cell surface, while the remainder is intracellular [126,127]. Activation of chemotactic receptors (eg C5a), or mechanical stimulation of the cell results in a rapid surface mobilisation of CR1 (and CR3) from the intracellular pool [127]. Limited cross-linking of CR1 via monoclonal antibodies or dimeric C3b induces attachment of the receptor to the cellular cytoskeleton, myosin dependent redistribution into caps and patches and is associated with the co-localisation of CR3 and FcR [128,129]. More extensive cross-linking with polyclonal antibodies, or with C3b and polyclonal anti C3, results in endocytosis of the receptor/ligand complex but no respiratory burst [126,130]. Induction of a respiratory burst and phagocytosis seems to require activation of the cell and may be achieved in monocytes by treatment with PMA [131] or by contact with the extracellular proteins fibronectin [132], laminin [133] and serum amyloid P [134]. These conditions also induce phagocytic activation of CR3, while treatment of monocytes with a T cell lymphokine results only in activation of CR1[131]. Treatment of phagocytic cells (PMN, monocytes and eosinophils) with PMA and a chemotactic peptide plus fibronectin [135] resulted in phosphorylation of CR1 and may suggest a role for protein kinase C.

Soluble CR1

Using ^{125}I labelled Fab MAb anti-CR1 (YZ-1), Yoon and Fearon [136] have demonstrated that human serum contains soluble CR1 (sCR1) with functional and structural properties identical to host ECR1. Levels of sCR1 correlated closely with ECR1 in normal donors, and represented about 7% of their numerical value. The origin of sCR1 is unclear. The fact that plasma levels did not rise, while ECR1 numbers fall, with duration of incubation of E *in vitro* in this study suggests that it is not released from E. However, it was also noted that the correlation between sCR1 and ECR1 was lost in SLE sera, and that the sCR1 levels were generally higher in pathological sera which may be taken to suggest the opposite conclusion . To date, no specific function has been found for sCR1.

Glomerular CR1

Binding of EC3b and EC3bi to to renal podocytes was recognised by Carlo et al in 1979 [137]. Kazatchkine and colleagues [138] used anti-gp205 in immunofluorescent studies of glomerular tissue from 75 patients with a variety of glomerular pathologies. CR1 was localised exclusively to the glomerular podocyte and was homogeneously distributed along the plasma membrane of this cell. Segmental loss of CR1 was found in a variety of renal conditions. The findings in SLE were variable and included normal staining in mesangial proliferative and membranous lesions, while the diffuse proliferative nephritis of SLE (but not other conditions) was associated with complete loss of staining. Renal CR1 does appear to have FI-cofactor activity (reviewed in [103]) and so may serve to limit complement activation within the glomerulus, but its exact role remains poorly understood and receptor loss is not directly correlated with glomerular C3 deposition [139].

1.45 Complement receptor type 2 (CR2)

CR2, the B lymphocyte C3d receptor, is a 140Kd single glycoprotein chain [140] and was first isolated from Raji lymphoblastoid cells (which express large amounts of surface CR2 and no other CRs) [141]. The developement of an anti C3d monoclonal with affinity similar to CR2 [142] allowed the demonstration of CR2 binding specificity to the C3d region of iC3b, C3dg, C3d and the presence of lower (100 fold) affinity for C3b. The function of CR2 as the B lymphocyte Epstein-Barr virus (EBV) receptor was demonstrated by the acquisition of EBV binding activity by *S. aureus* after linkage to CR2 [143]. The biological consequences of interaction between CR2 and its ligands are not fully understood but may be involved in the regulation of B cell proliferation. Cross linked C3dg can stimulate the transition of previously activated, synchronised murine B cells from G1 into S phase [144]. Treatment of B cell populations with polyclonal anti CR2 enhances certain proliferative responses [145], while CR2 becomes phosphorylated after treatment of B cells with phorbol myristate acetate (PMA) [135], suggesting the possibility of a role in trans-membrane signalling.

1.46 Complement receptor type 3 (CR3)

CR3 is an iC3b receptor on phagocytic cells and the lymphocytes that function in natural or antibody independent cytotoxicity (NK cells). CR3 is a heterodimer of CD11a and CD18 and shares an identical ß chain (CD18) with two other heterodimeric complement receptors, p150,95 (CD11c/CD18) and LFA-1 (CD11a/CD18)[146]. Genetically determined deficiency of this group of molecules has been found in a small number of individuals who suffer from recurrent infections, persistent leucocytosis, progressive periodontitis, ulcerating skin lesions and delayed umbilical cord separation [147]. Neutrophils from these patients show deficiencies in phagocytosis of iC3b bearing particles and a more

general defect in adhesion-dependent functions such as margination and migration. CR3 appears to have a complex lectin-like, calcium and magnesium dependent, binding site and may have specificity for sugar moieties in addition to complement binding (see [103] for review). This explains the affinity of CR3 for such diverse substrates as baker's yeast (*S. cervisiae*), rabbit erythrocytes and *S.aureus*.

1.47 Complement receptor type 4 (CR4)

The phagocytic cell C3dg receptor p150, 95 is the third member of the CD18 heterodimeric membrane glycoprotein family (CD11c/CD18) [146] and has similar binding properties to CR2. Designated CR4, p150,95 has affinity for C3dg and, to a lesser extent, iC3b and C3d [148]. Already described on neutrophils and macrophages, CR4 has also been recognised as a C3dg receptor on platelets [149].

Section 5
Complement, Erythrocyte CR1 and Immune Complex Processing in Primates

Introduction

The appearance of immunoglobulin and complement at the sites of inflammatory tissue damage has fostered the view that the role of the complement cascade in immune mediated diseases is wholly deleterious. However there is abundant evidence that complement has a number of protective functions, including the physiological processing of ICs. Complement thus plays a dual role in IC mediated tissue injury, it favours IC and antigen removal but if this fails, antigen persistence may lead to C-mediated tissue damage, further release of autoantigen and continued stimulation of the autoimmune process. The relationship between the phlogistic capacity of ICs *in vivo* and *in vitro* may not be direct, but the theoretical prediction would be that large, insoluble immune complexes are most likely to induce tissue damage if their formation and tissue deposition is uncontrolled. In general larger complexes activate complement more effectively and form at antibody equivalence, or at slight antibody excess. Complexes containing IgM may be strongly complement activating since IgM is itself polyvalent. Further aggregation of the complexed immunoglobulin may be induced by Fc interactions, rheumatoid factors or idiotype-antiidiotype binding. Complexes of antibodies with high affinity are more resistant to solubilisation by complement (see below), while larger polyvalent antigens also tend to promote larger complexes.

1.51 The interaction of complement with ICs

1.51A. Alteration of the physiochemical properties of the IC lattice

Classical and alternative pathway activation produces differing effects on the IC lattice depending in part on the phase of IC formation in which the interaction occurs. These effects are: inhibition of IC precipitation (IIP) and solubilisation of pre-formed ICs.

Inhibition of IC precipitation (IIP)

IIP was studied by Schifferli and co-workers [150-152] and is mainly dependent on early classical pathway components. Whereas complexes of soluble antigens remained in solution in the presence of normal serum, ICs formed in C1q deficient or EDTA-treated serum rapidly precipitated from solution [150]. Precipitation did not occur in Properdin deficient serum indicating that the alternative pathway was not involved in this complement function, but a secondary increase in the solubility of precipitated IC was attributed to

alternative pathway activity. The role of the C1 molecular complex was emphasised by the increased speed and degree of precipitation in the absence of this activity compared to deficiencies of C2 and C3. In addition, purified C1 is itself able to inhibit precipitation [151]. Clq binds to the Cγ2 domain of the IgG Fc and is thought to sterically interfere with Ig aggregation. Loss of this direct antiaggregational effect of the C1 molecular complex may contribute to the finding that nearly all individuals with deficiency of these components have an immune complex-like disease, compared with the lower incidence in C2 deficiency [153]. In contrast, Hong and colleagues [154] placed greater emphasis on the quantitative contribution of the covalent attachment of C3 to IIP. They also noted that the apparent molecular radius of complexes was dependent not only on the ratio of antibody to antigen (equivalence), but also on the absolute concentrations.

Solubilisation of preformed immune complexes

The alternative pathway may be activated by immune complexes. This may result from the presence of an activating antigen, or from alteration in the antigen surface which renders it so. Also, C3b can bind directly to the $F(ab')_2$ region of polymerised IgG which may form a protected alternative pathway convertase site. The result of the interposition of C3b molecules in the IC lattice is probably to reduce Fc-Fc interactions which would lead to further complex aggregation. The effect of the alternative pathway in solubilising ICs was described by Miller and Nusssenzweig [155]. In addition to the presence of C3, B, D and P, optimal solubilisation also requires the presence of Factors H and I [156] since the absence of these regulatory proteins allows fluid phase consumption of C3. Solubilisation is inhibited by the presence of rheumatoid factors [157].

1.51B.Opsonisation for erythrocyte processing and phagocytic catabolism

The cascade nature of classical pathway activation and the concurrent catabolism of activated complement components results in a dynamic process in which sequential complement fragments, with differing biological activities, are successively exposed on the IC surface. This "rolling" process probably allows optimal scope for complement mediated interactions between the IC and the variety of receptors on cells involved in IC transport and in antigen catabolism. ICs injected experimentally *in vivo* are rapidly taken up by erythrocytes via CR1 (see below). CR1 co-factor activity results in obligatory catabolism of IC-bound C4 and C3 to fully degraded particles. Deposited C4b will thus sequentially form a C3 convertase enzyme followed by catabolism to iC4b and bound C4d, while deposited C3b will in turn form iC3b and bound C3dg (some C3b may also remain unchanged in protected sites). Thus at any one time there may be a complex mixture of potential ligands on the IC surface though there will tend to be a predominant C3 fraction which is likely to determine the major receptor interaction.

The presence of C3b will mediate interaction with a wide range of CR1 bearing phagocytes, leucocytes and antigen presenting cells. Further catabolism to iC3b will cause release of the IC from the erythrocyte surface and direct binding towards CR3 bearing PMNs and monocytes. Finally, release of the C3c moiety leaves only surface bound C3dg (C4d) for which no major circulatory cell receptor exists, and ICs bearing only this complement fragment (such as erythrocytes in some cases of auto-immune haemolytic anaemia) may persist in the circulation for prolonged periods. However, stimulatory interaction with CR2 on B lymphocytes is still possible and may give these fully processed ICs a role in modulating the immune response. Moreover, at any point in this process, C1q and IgG may be bound via their cellular receptors, facilitated in activated phagocytic cells by co-capping of Fc and complement receptors.

1.52 The erythrocyte and immune complex processing

Immune adherence underlies a number of experimental methods for the recognition of IC. However, the potential relevance of this phenomenon to *in vivo* IC clearance has only recently been recognised. Medof and Oger [110] originally observed that serum treated ICs of ^{125}I-BSA/polyclonal rabbit or guinea pig anti-BSA would adhere to both erythrocytes and leucocytes in unfractionated mixtures of blood cells. The majority of ICs (80%) bound to erythrocytes due to their greater numbers, though the binding per cell was higher to leucocytes. This was followed by *in vivo* studies mounted by Cornacoff and co-workers [158] involving the constant rate infusion of large (10-288S) ^{125}I-BSA/anti-BSA complexes into the descending aortae of Baboons (and Rhesus monkeys). Cannulae placed in the hepatic, renal and portal veins and at other sites were aspirated to determine the distribution of ICs and their metabolism in various vascular beds. The complexes employed here are typical of those used in many *in vitro* studies. They were formed *in vitro,* at known equivalence, in the absence of complement and allowed to aggregate over 48 hrs prior to centrifugation and resuspension of the precipitate. They are probably optimal for complement activation and erythrocyte binding, but may not be representative of complexes formed *in vivo.* Interestingly, in two subsidiary experiments [158], no erythrocyte binding was achieved when these immune reactants were injected *in vivo* prior to IC formation. However, the findings of this study do in many ways represent the paradigm of erythrocyte IC processing and will be discussed in depth.

Maximal erythrocyte binding of an average of 61.2% (range 85.3-17.7%) of injected complexes was found to occur within 3 minutes of infusion. Remaining ICs remained associated with leucocytes and platelets in the "buffy coat" of the centrifuged haematocyte preparations. Removal of 73.1% of ICs from erythrocytes occurred within 5 minutes of infusion and was maximal in the hepatic circulation (69.3%). Levels of this early phase

removal from the splanchnic circulation (mainly splenic) were 26.3% and from the kidney were negligible (4.3%). Quantitation of tissue IC deposition was maximal in the liver, followed by spleen and lung. Those ICs bound to buffy coat cells were not significantly cleared in the time course of the experiment (30 mins). There was no evidence of erythrocyte loss occurring along with IC removal. Isokinetic sucrose density gradient ultracentrifugation (SDGU) of opsonised ICs remaining in supernatant after reaction with simian erythrocytes showed an almost linear relationship between size and erythrocyte binding. These experiments demonstrate clearly the potential efficiency of erythrocyte immune complex clearance mechanisms, though, as discussed above, may well represent the "best case" analysis.

In a second series of experiments of similar design [159], it was found that depletion of complement function by cobra venom factor (CVF, which stabilises the alternative pathway C3 convertase and depletes serum C3) and by heparin, resulted in failure of IC binding to erythrocytes *in vivo*, the ICs appearing to remain in solution in plasma. The clearance of these unbound ICs from the circulation was more rapid than control, erythrocyte bound complexes. In the complement depleted state the proportion of extra-hepatic clearance rose markedly. The inference from studies of this nature is that the rapid clearance of ICs to sites outside the liver and spleen represents pathological deposition at sites of tissue damage, though in these experiments, no direct evidence of renal deposition occured. In contrast [160], this group subsequently studied BSA/anti-BSA ICs formed with monoclonal antibodies of differing Ig class (IgG1 and IgA) and differing ability to fix complement and bind to ECR1. These antibodies were of comparable affinity, though the IgA was more anionic and the complexes formed were smaller. IgA IC bound less well to E and were cleared more rapidly from the circulation than the efficient E-binding/complement fixing IgG complexes. In these experiments, more careful anatomical examination did reveal increased deposition of the IgA complexes in the lungs and glomeruli, suggesting that reduced efficiency of primate erythrocyte IC clearance mechanisms may have pathological sequelae *in vivo*.

The first demonstration of similar IC erythrocyte binding of *in vivo* formed immune complexes in primates is the report by Edberg and colleagues [161]. Using polyclonal anti-dsDNA purified from pathological human sera, and radiolabelled high molecular weight DNA (ca 9000bp), these authors studied the behavior of DNA/anti-DNA IC formed *in vivo* in a primate (rhesus monkey) and non-primate (rabbit) model. Quantities of immuno-reactants used represented antibody excess, presumed to approximate to *in vivo* conditions. Purified γ globulin from normal donor serum was used as control. Injection of

antibody to give an experimental *in vivo* titre equivalent to 1/20 of the original patient's serum titre was followed by rapid infusion of the radiolabelled antigen. Considering the primate experiments. Under these conditions >90% of the infused DNA formed complexes and was precipitatable by excess anti-human immunoglobulin. Maximum binding of these *in vivo* formed ICs to erythrocytes was 45% and occured within 90 seconds of infusion. In addition, in the rabbit experiments, it was found that this quantity of antibody was sufficient to mediate complexing of a second infusion of DNA 1 hr after the first. The authors consider that this fact demonstrates the lack of cross-reactivity of these high binding dsDNA autoantibodies *in vivo*, as opposed to their behaviour in certain *in vitro* studies.

Paccaud, Schifferli and co-workers have studied the behaviour of tetanus toxoid-anti-tetanus toxoid immune complexes (TT/anti-TT IC) *in vitro* and *in vivo* including human studies. Initial studies [162] have clarified the properties of this model immune complex. ICs formed in 25x antibody excess, and centrifuged to remove aggregates, are predominantly 45s in size. When incubated in serum the Mr of these complexes increased, a change which was abolished by EDTA and was probably due to the binding of C3. Abrogation of binding to E treated with trypsin (which hydrolyticaly cleaves CR1) or with anti-CR1 antibodies confirmed the specificity of the IA reaction. The binding of these complexes to human E *in vitro* was rapid, near maximal binding occurred within 1-5 minutes and minimal further binding took place over the succeeding 30 minutes. Approximately 45% of administered complexes were bound, and SDGU of subsequently released complexes showed these to have increased in size suggestive of complement binding as above.

Studies with experimentally depleted sera and in C4 deficient guinea pigs *in vivo* demonstrated the classical pathway dependence of these IA reactions. Sera depleted of C1q, C2 and C4 were unable to promote erythrocyte IC binding, while replenishment of the deficient factor restored both binding and the kinetics of the reaction. It is of interest to note that reconstitution of 10% normal C1q and 1% normal C4 was sufficient to normalise IC binding. As with other experiments, clearance of pre-formed CICs *in vivo* was more rapid in the absence of classical pathway activity (in the C4 deficient guinea pig), while CR1 mediated binding (to platelets in this species) and slower clearance resulted from the infusion of C4.

The choice of TT as antigen has allowed its use in human studies. Tetanus toxoid is a denatured protein which has been extensively used *in vivo* and can be considered to have no toxicity. Anti-TT antibodies, purified from the serum of hyperimmune subjects is

commercially available and suitable for *in vivo* use and, lastly, no clinical effects were noted as a result of infusion of these complexes in the animal experiments described above. Schifferli and others have reported the use of these complexes *in vivo* in 3 normal human volunteers and in 3 patients with SLE [163]. TT/anti-TT complexes were injected intravenously either free (in a mixture with 99mTC erythrocytes, series 1), or after *in vitro* binding to autologous 99mTC erythrocytes (series 2), in separate experiments on successive days. Analysis of samples withdrawn at intervals between 1 and 60 minutes allowed computer modeling of clearance kinetics of IC from the circulation. Separation of complexes into fractions by SDGU prior to incubation with E corroborated the close correlation of IC size with erythrocyte binding *in vitro* and rapid binding of large complexes within 1 minute was noted.

In normal controls, the clearance of IC was saturated during the first minute, while in the 3 subjects with SLE, 25.3, 19.5 and 22% of injected complexes were lost from the circulation within the first minute. This early removal from the circulation is referred to as IC "trapping". After the first minute, clearance of IC can be described by a monoexponential model in both groups and showed a mean clearance of 11.2%/min in the normal controls. Whereas rapid binding of IC to E was noted in the first series of experiments, the opposite, release of IC, was noted in the 2nd series where *in vitro* binding to E had taken place prior to injection. Both the levels of uptake and release from erythrocytes in the first minute after injection showed a relationship to number of ECR1/cell, which varied in each subject. No significant loss of 99mTc labelled E occured during the period of these experiments, implying that alterations in IC levels were independent of RES removal of E. It is of interest that the rate of clearance of ICs noted in this study were similar to those observed for DNA/anti-DNA complexes in the baboon, described above, and that once bound to erythrocytes, clearance of IC was similar in both SLE and control subjects. These data appear to support the conclusion that alterations in the initial, rapid binding of IC to E may be the one of the most fundamental differences in immune complex handling in SLE, in so far as these large, soluble, complement fixing ICs are representative of those formed *in vivo*.

Davies and co-workers have had the opportunity to study the *in vivo* formation of immune complexes in patients receiving immunoradiotherapy for ovarian malignancy [164]. On day 1, patients received intraperitoneal injection of ^{131}I mouse anti-tumour antibody at the therapeutic activity of 10mCi/mg. In order to increase the clearance of these therapeutic antibodies and to reduce marrow toxicity, patients are then administered human anti-mouse IgG on day 2 and 3. Infusion of ^{125}I labelled second antibody allowed the kinetics of *in vivo* IC formation and clearance to be studied. Many of the observed sequelae were those

predicted from the pre-formed complex models, though the quantitative importance of erythrocyte related mechanisms was less in this model. Immune complexes of 19-40S (by SDGU) were formed within 5 minutes of the injection of the second antibody and were cleared to the liver with a half life of approximately 11 minutes. Circulating complexes were measured by 4% polyethylene glycol (PEG) precipitation and by solid phase C3d and C1q binding assay. Between 8 and 11% of the total complexed material was found to bind to E (via CR1), though the maximal fractional erythrocyte complex binding was higher at 25% of the complexed material in the circulation at a point soon after the peak of complex formation. Coincident with immune complex formation and clearance, a 47% fall in serum C4, C3 and CH50 occured and at the same time a rise in erythrocyte surface C4 and C3 (to 1230 and 2590 molecules/cell respectively) was found. In the 20 minutes after IC formation a 32% fall in E CR1 numbers was measured. These changes resemble the abnormalities seen in SLE erythrocytes which include raised cell surface complement deposition and low CR1 (see Chapter 1 section 6) and suggest that such abnormalities may arise, in part, as a result of erythrocyte participation in immune complex transport.

The localisation of exogenous ICs to the liver (and to lesser extent the spleen) has repeatedly been shown, and the original experiments of Comacoff demonstrated removal of IC from erythrocytes in the hepatic circulation presumably by the fixed phagocytic cells of the RES. Recent data has demonstrated that E-CR1 binding of ICs increases the efficiency of phagocytosis *in vitro* [165]. Phagocytic uptake by the human monocyte cell line U937 was compared between opsonised test ICs (heat aggregated IgG) in solution and bound to E-CR1. The transfer of E bound ICs occured within 2 minutes, whereas removal from solution required up to 1 hour to reach the same steady state reduction. The erythrocytes themselves did not bind to U937, and erythrophagocytosis did not occur.

1.53 Erythrocyte CR1 IC binding and release

CR1 binds opsonised ICs via C fragments, and is also involved in the catabolism of these fragments to particles for which it has no affinity. The process of IC binding must therefore be followed by release of the complex with subsequent rebinding to other E-CR1 molecules either because of the persistence of C3b fractions, or as a result of fresh C activation. The net result of this ongoing binding and catabolic release is two-fold. Firstly, it may allow release of the complex from the erythrocyte surface to facilitate other cell receptor interactions, such as phagocytosis by hepatic Kupffer cells. Secondly, the reactive complement species, mainly C3b, iC3b and C4b which opsonise the complex are fully catabolised by CR1 (and FI, FH, DAF and C4bp) to C3dg (and C4d), which has no further potential to provoke inflammatory interactions, and will therefore be of lower

pathogenicity if the complex escapes phagocytosis and is deposited in tissue. This model has been supported by direct observations [166] and analysis of the kinetics of the interaction of 3H DNA/anti-DNA ICs with SLE erythrocytes [167]. This last study also demonstrated that low CR1 number and not any alteration in binding function was the prime determinant of reduced IC binding by SLE erythrocytes.

Summary

A model of primate IC processing has now emerged. Immune complexes form *in vivo* in the presence of complement inducing C activation and deposition in the evolving IC lattice. This modulates the IC lattice to maintain solubility and to reduce lattice extension. C3b (and C4b) bearing IC are bound by ECR1 to the E surface. Fluid phase Factors I and H, with CR1 as co-factor catabolise the C fractions allowing release of the IC. Once released, the IC bearing catabolised C may bind to B cells or phagocytes depending on the dominant C fraction (and other ligands) present, or further C deposition may occur allowing rebinding of the IC to E. The erythrocyte thus acts as a "shuttle" for ICs in the circulation and takes part in the catabolism of cell surface opsonic C molecules. In the proximity of the RES of the liver and spleen, release of the IC will allow its uptake by phagocytes, which may be activated by the presence of a variety of IC borne C fragments or Immunoglobulin. This leads to the safe catabolism and disposal of antigen. During this process E suffer bystander C3 and C4 surface deposition Further, it is proposed that release of enzymes during phagocytosis of receptor bound IC leads to proteolytic cleavage of CR1.

Section 6
Reduction of Erythrocyte CR1 in SLE

Introduction

Patients with SLE have reduced expression of erythrocyte CR1 numbers which is hypothesised to contribute to disease susceptibility by reducing the efficiency of E-CR1 dependent IC clearance. Both acquired and inherited factors have been postulated to explain this finding, though the bulk of evidence appears to point to acquired deficiency as the dominant process in the majority of cases. The mechanisms of CR1 reduction are partially understood. Loss of CR1 is also seen in other diseases characterised by the presence of immune complexes, and one mechanism of E-CR1 reduction is likely to be hydrolytic cleavage of the receptor during the phagocytic removal of bound ICs in the hepatic RES. In this thesis, I will present data which suggests that direct erythrocyte binding of anticardiolipin or related autoantibodies may be an additional pathogenetic mechanism.

1.61 Reduction of CR1 in SLE: evidence for inherited factors

Reduced erythrocyte CR1 numbers in patients with SLE have been detected by a variety of different methods including reduced immune adherence haemagglutination activity (IAHA) [168] and direct enumeration of E-CR1 by radioimmunometric assay [169].

The possibility that this reduction was inherited was examined by Wilson and colleagues [170] in a study of CR1 numbers amongst 38 patients with SLE, 47 of their 1st and 2nd degree relatives (in 6 pedigrees), and 113 normal controls in Boston. Monospecific F(ab´)2 anti-C3b were used in radioligand binding assays of E-CR1 numbers, along with assessment of dimeric C3b binding sites and serological data. Linear regression of anti-CR1 and dimeric C3b binding site numbers with high correlation, suggested that 9 antigenic sites existed for each functional C3b site in both patients and controls. This polyvalent binding contributes to the high overall estimates of CR1 numbers which are systematically greater than those seen in these patients using monoclonal antibodies (see below). Alternatively, it is not clear in what way background (non-specific) binding of antibody, or decay of label were corrected for in the assay method. Each of these factors would contribute to raised estimates of CR1 numbers but would probably not otherwise deform the data presented. Mean CR1 numbers were were 5014 +/- 15/E in normal controls and were significantly lower (2804+/-241) in subjects with SLE. Analysis of the frequency distribution of CR1 numbers in the control population showed a trimodal distribution, and family studies suggested co-dominant expression of alleles for High (H)

and Low (L) giving 3 haplotypes (HH), (HL) and (LL) with associated ranges of CR1 numbers. Evidence for a genetic basis of CR1 reduction in SLE patients comes from analysis of mean E-CR1 numbers in 47 relatives of 6 probands. These clinically normal individuals had 3167+/-196 CR1/cell and were statisticaly different from both unrelated normals and the 6 probands (2049+/-94 CR1/E). By comparison, the spouses of SLE patients were lower than, but statistically inseparable from, the normal range with 4528+/-484 CR1/E.

There is little doubt that this study describes the gene linked distribution of ECR1 numbers (numerical polymorphism of CR1, see below). It is also apparent that the relatives of certain patients with low CR1 numbers also have low CR1 numbers, and that this is suggestive of an association of the proposed LL or HL genotype with the disease state. However, the ranges of CR1 numbers seen in each group and within each part of the proposed trimodal (gene associated) frequency distribution overlap so extensively that the accurate ascription of genotype from phenotype in any individual is not possible. It is notable that the SLE probands whose families were studied show CR1 numbers at the lowest end of the normal range and it is therefore possible that an unrepresentative sample of homozygous low CR1 (LL) SLE patients were inadvertently studied.

However, these same subjects were studied a second time 4 years later [171] and on this occasion, direct analysis of genotype and measurement of CR1 number by monoclonal antibody (Yz-1) were performed. It was observed that CR1 numbers estimated by Yz-1 correlated with those previously observed in the subjects retested and thus appeared to be a stable trait. This has been interpreted as evidence for the predominance of inherited control in the determination of CR1 expression (see [101]). CR1 numbers in patients, relatives and normal controls were 281 (+/-34, SEM), 457 (+/-21) and 553 (+/-21) respectively. The 6.9 (low expression) and 7.4kb (high expression) Hind III RFLP fragments associated with CR1 numerical polymorphism have already been described. The 0.75-kb cDNA probe CR1.1 was used for Southern blot analysis of genomic DNA in a proportion of the patients, relatives and controls analysed in this 2nd study. The major findings were that the frequency of the allelic polymorphisms were not equal amongst the experimental groups. Figure 9 (overleaf) is redrawn from the original data and shows that the homozygous 7.4-kb (HH) haplotype is underrepresented in the patients and their relatives. Mean CR1 numbers associated with each genotype was lower in *both* patients and relatives than in controls sharing the same genotype and CR1 numbers in family members showed a statistically significant regression correlation with the CR1 numbers of disease probands [101]. The conclusion reached is that the data support an important genetic contribution to reduced CR1 in SLE.

However, certain aspects of the data may not be fully consistent with this hypothesis. The homozygous low condition was infrequent in all groups, but was less frequent in the patients than in the control groups. This may suggest that the LL genotype is lethal in the presence of SLE, but is otherwise not easy to explain if the allele is a disease susceptibility gene. Moreover, the reduction of the HH genotype was more marked in the patients' relatives than in the disease group (see figure 9). Lastly, the occurence of reduced CR1 in the genotype matched relatives when compared with controls argues for the existence of an associated second genetic (or acquired) abnormality in these individuals. No such second locus has been described. However, there is further evidence of a genetic influence in phenotypic studies of patients from Japan [168].

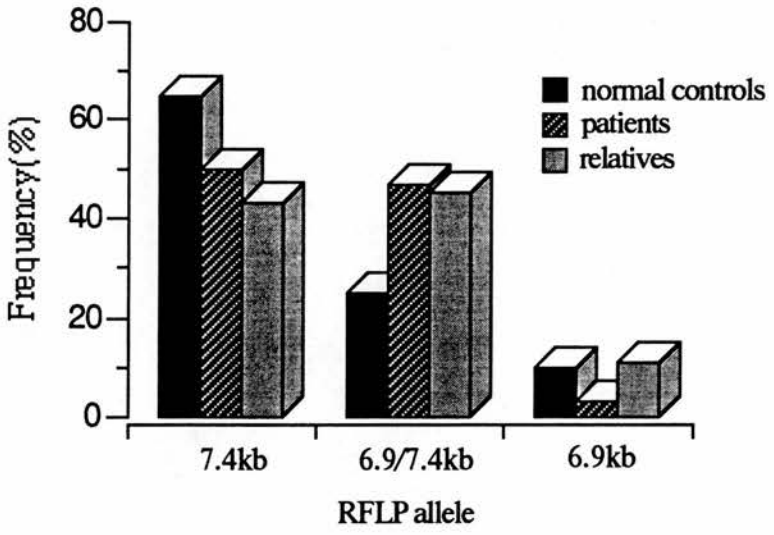

Figure 9. Frequency of RFLP alleles for high (7.4kb) and low (6.9kb) ECR1 numbers in SLE patients, their relatives and normal control subjects in Boston.

By comparison, a family study and further genetic analysis of patients in Cambridge did not support the conclusion that reduced CR1 numbers are inherited in SLE, nor did analysis of CR1 numbers from SLE families in Athens [101]. Walport and colleagues [172] employed E11 [173], a murine monoclonal anti CR1, to enumerate erythrocyte CR1 sites in 48 SLE patients and 48 consanguineous relatives from 17 families. The measured range of CR1 numbers in these normal relatives was 145-1,214 CR1/E and a reduction of CR1 in SLE probands was seen. An attempt was made to fit family data to a Mendelian model of inheritance, as proposed by Wilson, but proved impossible due to difficulty in determining the ranges of CR1 number appropriate to each genotype. In addition, certain families are described in which the SLE propositus has phenotypically low CR1 in families

who seem to be genotypically high. These data are taken to exclude genetic factors as the "sole" source of phenotypic variation in SLE patients, though they do not exclude a superimposed acquired CR1 loss, which may have obscured other effects.

Data derived from direct observation of gene frequency, however, seems compelling and appears to exclude inherited reduction of CR1 as a general disease mechanism in SLE [174]. The 6.9 (low expression) and 7.4kb (high expression) Hind III RFLP and the CR1.1 partial cDNA ligand were used to probe genomic DNA in a family study of SLE patients, their consanguineous relatives and unrelated normal controls. Moldenhauer's data demonstrate that the inheritance of these 2 alleles is co-dominant with gene frequencies of 0.73 for the 7.4kb (H) band and 0.27 for the 6.9kb (L) band. Mean CR1 numbers were 725 CR1/E (95% CI; 652-801) in 29 normal subjects with the HH haplotype, 463 (410-520) in 27 with the HL bands and 260 (160-383) in 4 control subjects with the homozygous low genotype. The gene frequencies found in SLE patients were not significantly different to those in normals, but within each genotype, CR1 numbers for patients were lower than both relatives and controls, while relatives were not significantly different from controls. In this study only 4 subjects with the homozygous low genotype were detected and none had SLE. The authors conclude that the inherited variation in CR1 numbers does not play a role in causing susceptibility to SLE. To date, it has not been resolved whether these mutually exclusive conclusions reflect true differences in disease mechanisms amongst different populations.

1.62 Acquired reduction of ECR1 and associated erythrocyte C deposition

While the role of genetic factors remains controversial, there is abundant evidence that loss of CR1 may be acquired in patients with SLE [175-178]. CR1 numbers have been found to fluctuate with disease activity [175-177] and transfusion of E with high CR1 numbers into SLE patients has allowed direct observation of acquired CR1 reduction *in vivo* [209]. In these experiments, group A Rh(D) +ve patients were transfused with type O Rh(D) +ve cells while O Rh(D) +ve patients received O Rh(D) -ve blood. Differential agglutination allowed retrieval of transfused cells which had acquired a reduction in CR1 numbers in two patients with SLE and one with cold agglutinin disease [179].

A number of studies associate ECR1 reduction with cell surface C3 deposition and suggest that C-binding may result from erythrocyte IC binding. Inada et al [178] found that the presence of IC activity in serum correlated with defective C3b receptor function of SLE E. Ross and colleagues [175] observed an inverse relationship between E-CR1 and erythrocyte bound C3dg which varied with disease activity while *in vitro* experiments demonstrated that approximately 250 C3dg molecules/E were deposited subsequent to the

binding and release of soluble DNA/anti-DNA immune complexes to E in whole blood. In the transfusion experiments conducted by Walport and colleagues [179], there was concomitant deposition of C3dg on E as CR1 loss occured. CR1 reduction and E C3dg deposition was also noted in patients with other diseases associated with C activation such as auto-immune haemolytic anaemia (AIHA), mycoplasma pneumonia, PNH and chronic cold agglutinin disease (see [175]), and the experiments of Davies and colleagues [164] confirm the association of *in vivo* IC formation with E surface complement deposition and CR1 reduction. The mechanism which links E-surface complement deposition and CR1 loss seems likely to be related to binding to phagocytic C3b receptors with subsequent release of proteolytic enzymes (see below).

1.63 Functional correlates of ECR1 reduction and abnormalities of C-mediated IC clearance in SLE

The importance of CR1 reduction in immune complex disease lies in the possibility that this may result in reduced efficiency of the primate erythrocyte IC clearance mechanism. Taylor et al [180], demonstrated reduced C-mediated binding of ^{3} HDNA/anti-DNA ICs to SLE erythrocytes *in vitro*, though no alternative enumeration of CR1 on these E was obtained. Further kinetic studies comparing the binding and release of these complexes to normal E and those from SLE patients with low C-mediated binding were undertaken [181]. Normal E bound these ICs within 4 minutes at 37°C, while SLE erythrocytes both bound less IC and reached equilibrium after 30 minutes. Confirmation of defective CR1/ C-mediated IC clearance in SLE patients *in vivo* was obtained by Schifferli and colleagues using the TT/anti-TT IC model described above [182]. Briefly, nine controls and 15 patients with conditions characterised by IC formation or hypocomplementaemia, including 2 with hereditary C deficiency were studied. Radiolabelled IC were injected free or, in a second series of experiments, after binding to E in the presence of normal serum *in vitro*. Two phases of IC clearance were identified with free IC, an initial rapid phase occurring within the first minute, prior to circulation of the IC to the hepatic MPS [163] was referred to as "trapping". Thereafter, a slower phase of IC removal with monoexponential kinetics occured. Trapping was thought to take place before the IC could have been exposed to the hepatic RES and may thus represent pathological deposition of IC in target sites. It was seen predominantly in the patient group (and 1 control subject) and correlated with low CR1 number and low binding of IC to E within the 1st minute of infusion. The monoexponential clearance of IC occured at a mean rate of 14.1%/min, was faster in the patients than controls and showed an inverse correlation with CR1 numbers and the retention of IC by E after 1 minute. The second series of experiments showed that up to 81.4% of IC were released from E within 1 minute *in vivo* and that release was inversely correlated with CR1 number. A small proportion of released ICs showed trapping in

patients with low CR1 and a high percentage release of IC. The complement deficient patients were unable to opsonise ICs for E binding *in vivo* and showed trapping and rapid second phase clearance. However, in these, as in the other patients, infusion of IC bound to E *in vitro* abolished these abnormalities and led to only slightly more rapid second phase clearance than was observed in normal controls. In one patient with inherited C1q deficiency infusion of 300ml of fresh frozen plasma restored 29.8% of IC binding to E and 5% haemolytic CH50 (personal observation).

These experiments demonstrate the potentially pathogenic role of hypocomplementaemia and reduced CR1 in patients with IC mediated diseases, but can be criticised on the basis of the size and antigenic composition of the model IC used which may be unlike those found *in vivo*. Certainly, the characteristics of the IC model appears to affect the abnormalities found. Lobatto *et al* defined the clearance kinetics of soluble HAGG ICs [183] in SLE and control subjects. The patients showed *slower* clearance of IC in the presence of reduced E binding and complement activation. This was attributed to reduced splenic uptake of these ICs by external scintigraphy, and was thought to represent splenic Fc receptor dysfunction previously characterised in relation to E-IgG complexes [184,185].

Inhibitor proteins may further reduce C-mediated IC clearance mechanisms in certain diseases. Ng and colleagues [186] found a heat-labile protein with lysine-binding properties which inhibits CR1 binding of opsonised ICs in the sera of a percentage of patients with SLE. The inhibitor was not endogenous ICs, RFs, serum protease enzymes or plasminogen. A second inhibitor, which interferes with IC opsonisation and C-mediated inhibition of precipitation by displacing C1q from its Fc binding site, has recently been identified in RA and in normal control sera and is a 60-kd glycoprotein [187]. The role of these, or other, inhibitors in the pathophysiology of IC related diseases has not been defined.

1.64 Mechanisms of ECR1 loss: proteolysis and association with positive Coombs' test

A number of mechanisms may underlie the acquired reduction of CR1 in SLE. CR1 is susceptible to cleavage by tryptic enzymes [188] which may be produced by peripheral blood leucocytes (PBL) or the phagocytes of the fixed mononuclear phagocytic system [179]. The deposition of C3dg which was induced *in vitro* by DNA/anti-DNA IC [167] was not associated with CR1 reduction despite the presence of high numbers of PBLs. Alternatively, immune complexes are removed from the E surface during transport through the circulation of the liver and spleen [158]. Endocytosis of IC receptor by hepatic Kupffer cells (reviewed in [102] and [179]), or "bystander" tryptic cleavage due to release of

proteolytic enzymes, could occur during this process. Cell-surface complement, in the absence of bound ICs would promote similar effects. Blockade of receptor by ICs or fixed C3 fragments have been discounted as possible mechanisms, as have the effects of erythrocyte aging [175,189] during which CR1 numbers gradually decline [188]. Autoantibodies to CR1 have been defined in the sera of patient with SLE. The presence of anti-CR1 was associated with a fall in measurable E-CR1 sites and a flare in disease activity, all of which reversed with treatment [190]. However, such antibodies appear to be rare, occurring in 1% of 179 SLE sera tested [191].

An association between reduced CR1 activity (IAHA assay) and a positive Coombs' test has been noted [192]. This was attributed to receptor occupancy by C3c molecules or immune complexes, although this seems an unlikely explanation in view of the evidence for IC binding and release, or the known co-factor activity of CR1. A subsequent report links the presence of antiphospholipid antibodies (aPL), detected as anticardiolipin (aCL) binding, with hypocomplementaemia and a positive Coombs' test [193]. Both serum C3 and C4 levels were correlated with aCL, but no association was found with laboratory (DNA binding, mean CIC levels), or clinical, indicators of disease activity. Moreover, aCL binding activity could be eluted from the erythrocytes of one Coombs' positive patient, suggesting that a mechanism explaining the association may be direct binding of aCL to E, with subsequent classical pathway activation. Deléze and colleagues [194] have reported an association between the rare syndrome of combined AIHA and thrombocytopaenic purpura (Evan's syndrome) and aCL in patients with SLE, underlining the potential role of these autoantibodies in the haemocytopaenias of immune disease. In this thesis I will present data which suggest that aCL (or related specificity) may also be associated with reduced CR1 numbers [195] and that aPL may be common anti-erythrocyte autoantibodies [196]. I will also present data which suggest direct binding of polyclonal aCL to erythrocytes and that aPL in Coombs' positive subjects show increased binding to neutral PLs.

Chapter 2. General Methods

2.1 Buffers

The following standard buffers were used. All chemicals were supplied by BDH Chemicals Ltd, (Chadwell Heath, Dagenham, Essex) and were ANALAR grade unless otherwise stated.

Phosphate buffered saline (PBS), pH 7.4

potassium dihydrogen phosphate 0.1M (38.6mls),
dipotassium hydrogen phosphate 0.1M (160mls),
NaCl 16.35g made up to 2lts in double distilled H_2O (ddH_2O)

Complement fixation test diluent (CFD)

Oxoid, Unipath Ltd, Wade Rd, Basingstoke, Hants.

Ethylenediaminetetraacetic acid (EDTA) 0.2M:

500mls 0.2M Na_2 EDTA (37.22g)
0.2M Na_4 EDTA($4H_2O$) (41.62g) made up to 1ltr in ddH_2O

2.2 Purification and Preparation of Monoclonal Antibodies

Monoclonal and other antibodies were the kind gift of a number of workers and were provided in differing media, as listed below in table 5, below and overleaf.

Antibody	Ligand	Species	Source/Medium
Anti-Rodgers:1 [197]	C4A	Murine	Dr. C. Giles and Ms T. Robson, RPMS, London. Murine hybridoma cell culture supernatants
Mab 1228	C4B	Murine	Prof Rittner, Mainz, GDR. Ascites
Mab T2. C5.12	C4d	Murine	Prof G. Ross, S.Carolina, USA. Ascites
Clone 3 [198]	C3d	Rat	Prof P.J. Lachmann MITI, Cambridge. Purified antibody
Clone 4 [198]	C3c	Rat	Prof P.J. Lachmann MITI, Cambridge. Purified antibody
E11 [173]	CR1	Murine	Dr N. Hogg ICRF, Lincoln Inns Fields, London. Purified antibody
R340	Mouse IgG	Rabbit	Dr M.J. Walport, RPMS, London. Polyclonal antiserum

Antibody	Ligand	Species	Source/Medium
Anti-human C4	C4	Sheep	Serotec laboratories, Oxford. Polyclonal antiserum
Anti-DAF [8]	DAF	Murine	Dr E Medof, NewYork. Polyclonal immunoglobulin
Mab BH1 [199]	Phospholipids (-ve charge)	Human monoclonal	Dr C. Mackworth-Young, RPMS, London and Dr K.Thompson, MITI, Cambridge. Cell culture supernatant

Table 5. List of antibodies employed.

A number of procedures, as follows, were used to prepare the these reagents prior to use:

2.3 Cell culture of MAb Anti-Rodgers:1 hybridoma cells

Murine hybridoma cells were provided by Dr C.Giles and Ms T. Robson, stored in liquid nitrogen with DMSO preservative. Conventional cell culture techniques were used throughout to grow these cells and to harvest supernatant. All procedures were performed in a laminar flow sterile hood using no-touch sterile technique.

Media: Full RPMI 1640: (Sigma)
Cell culture medium: to 170mls ddH_2O add: 20 mls 10 x RPMI, 2mls penicillin/streptomycin (10,000IU/ml), 2mls L Glutamine (0.2M), 2.5mls Na Pyruvate (11mg/ml), 6.5mls $NaHCO_3$ (7.5%), 20mls heat inactivated foetal calf serum (FCS)

Method

Briefly, the following steps were followed.

1. Mouse splenic macrophages were harvested from a recently sacrificed Balb/c mouse (kindly provided by Ms J. Roberts, Dept Immunology, RPMS) and cultured for 48hrs in full RPMI medium in a 25 ml flat-walled cell culture flask to provide a macrophage lawn. These were examined for morphological evidence of infection and viability prior to proceeding.
2. Frozen hybridoma cells were thawed rapidly in a 37°C water bath and 5mls of FCS added dropwise with agitation prior to placing in a sterile 25 ml flask with macrophage "feeder" cells as above.
3. Cells were washed twice in 20 mls cold culture medium by centrifugation at 1200 RPM for 5 minutes, resuspended in 20mls fresh culture medium and added to the macrophage

lawn in 25ml culture flasks at $2.5x10^5$ cells/ml culture medium.

4. Cells in culture were incubated at 37°C in a sterile incubator in air and 5% CO_2.

5. Medium was changed and harvested after 1 week of growth and at 48hr interval thereafter or sooner if the indicator colour altered.

6. Supernatants for IgG purification were obtained after 12 days of culture and aliquots of growing cells were taken and stored in liquid nitrogen in DMSO for future use.

2.4 Affinity Purification of monoclonal anti-C4A, anti-C4B and anti-C4d

Purification of Mab anti-Rodgers:1 (anti-C4A) culture supernatant, Mab 1228 (anti-C4B) and Mab T2. C5.12 (anti-C4d) ascites was by acid elution from protein A affinity column after binding in the presence of high salt concentration at alkaline pH. These conditions have been shown to be optimal for the purification of IgG murine monoclonal antibodies [200]. All antibody purification was carried out at 4°C in a cold room.

Materials

Affinity column: A 1cm x 5cms column (bed volume approx 4mls) of protein A-conjugated Sepharose Cl-4B (Pharmacia, Midsummer Boulevard, Milton Keynes) was kindly donated by Dr. R. Harrison, MITI, Cambridge and kept in storage buffer at 4°C prior to use

Peristaltic pump and tubing

Amicon (Amicon LTD, Upper Mill, Stonehouse, Glos) ultrafiltration apparatus and pressurised nitrogen gas cylinder

Buffers

1. Column storage buffer: PBS, 0.5% Na Cl, 10mM NaN_3 , pH 8.0
2. Regeneration buffer: 0.2M Glycine HCl, pH 2.7
3. Binding buffer: 1.5M glycine, 3.0M NaCl, adjusted to pH 8.9 with 5M NaOH
4. Elution buffer: 100mM Citric acid, adjusted to pH 3.0 with 5M NaOH
5. Dialysis buffer: PBS, 10mM NaN_3, pH 7.4

Method

1. Samples were first equilibrated with loading buffer, small volumes of ascites were diluted to 50% (by volume) in 2 x loading buffer (anti-C4B and anti-C4d), while larger volumes (anti-C4A cell culture supernatant) were equilibrated by the direct addition of NaCl and glycine. pH was assessed by pH meter or by paper pH strips depending on the sample volume.

2. The column was washed extensively first with PBS pH 7.4, then with acid glycine

regeneration buffer until the optical density at 280nM (OD_{280}) of eluate was zero.

3. The column was then adjusted to pH 8.9 by washing in loading buffer and the sample loaded by continuous, recycled perfusion using a peristaltic pump at 4°C for 18hrs.
4. The column was then washed in PBS, pH 7.4 till the OD_{280} was zero.
5. Aliquots of each sample and the column exclusion fractions were retained.
6. Bound material was then eluted by perfusion of the column with elution buffer. The pH and OD_{280} and of 1ml fractions was recorded and collected until the OD_{280} had returned almost to zero.
7. Fractions were then assayed for IgG by Ouchterlony gel immunoprecipitation.
8. Resulting IgG containing fractions were pooled and dialysed against 10 litres of PBS, NaN3 10mM at 4°C for 24 hrs prior to concentration using an Amicon filter at 20lbs/inch^2 of Nitrogen followed by final estimation of OD_{280}.
9. In the case of anti-C4A purification: pre-loading, column exclusion fractions, pooled 1st protein peak and a sample from the elution tail were tested for anti C4A, C4B and C4d activity by agglutination of C4 coated erythrocytes (kindly performed by Dr C. Giles, RPMS).

For anti-C4d, radiolabelled purified IgG was tested by co-precipitation with commercial anti-human C4. Briefly, whole serum was placed in 3 wells cut in 0.7% agarose (10µl/well) and subjected to electrophoretic separation. Two troughs were then cut in the gel between the wells. One was filled with mixed ^{125}I anti-C4d and cold anti-C4, the other with cold anti-whole serum. The presence of specific anti-C4 activity in the radiolabelled antibody was confirmed by comparison of immunoprecipitation in stained gel and autoradiography of the labelled antibody precipitation.

Results

Active antibody was eluted in the first protein peak in fractions 4-8 and appeared as the pH of the eluate fell from 8.9-3.0. The representative acid elution profile of anti-C4d is shown overleaf in figure 10, while representative profiles for the purification of anti-C4A and anti-C4B are shown below in figure 11.

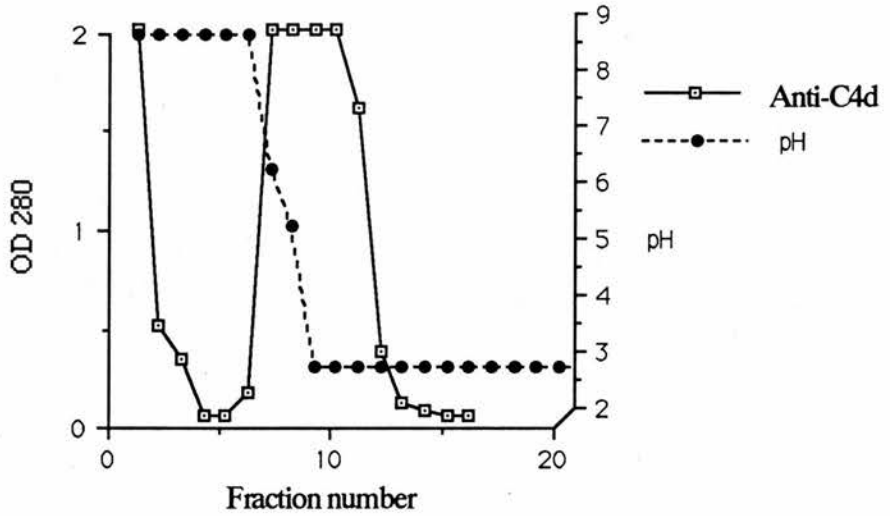

Figure 10. Acid elution profile for the affinity purification of anti-C4d ascites.

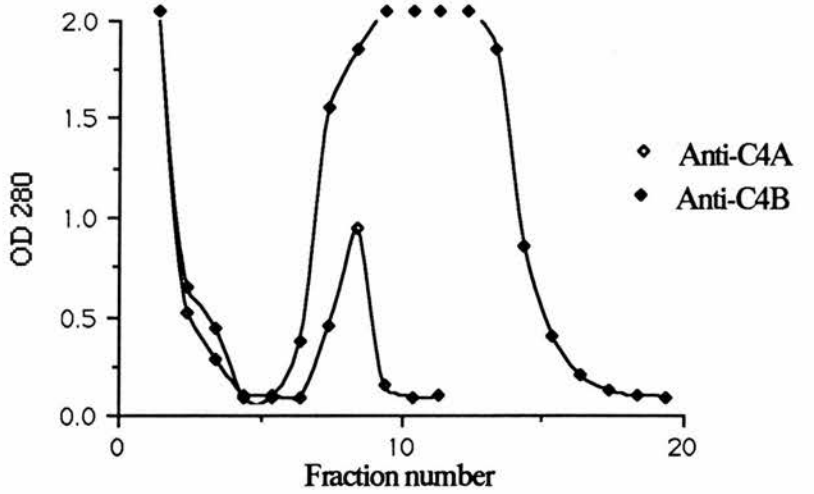

Figure 11. Representative acid elution profiles for the affinity purification of anti-C4A cell culture supernatant and anti-C4B ascites.

The presence of IgG the first peak in column fractions was confirmed by Ouchterlony gel precipitation for each antibody. For anti-C4A, binding activity and specificity of cell culture supernatants, column void volume, pooled 1st peak IgG and post affinity column supernatant were tested in a semiquantitative fashion by their ability to agglutinate erythrocytes coated with C4 from donor serum of known Chido and Rodgers serotypes.

Agglutinating activity was present in cell culture supernatant (1+) and was highest in pooled IgG fraction (+++). No agglutination was found in supernatant after affinity absorption and no agglutination was found in any fraction in the presence of C4B (Ch:1 +ve, Rg:1 -ve) bearing erythrocytes. For anti-C4d, co-precipitation of C4 from whole serum by radiolabelled purified protein and commercial anti-human C4 confirmed the activity of the antibody preparation.

Lack of cross-reactivity of each anti-C4 antibody was demonstrated in sensitive radioimmunoassay by reaction of each with genetically deficient serum, ie anti-C4A recognised C4B deficient serum but not C4A deficient, while the opposite was true of anti-C4B. Anti-C4d recognised both C4A and C4B deficient sera but not C4d deficient serum (this rare serum was kindly donated by Dr C.Giles, RPMS). No cross-reactivity was found with serum at 1:100 dilution (approx 2.4μg/ml), while the assay was capable of recognising C4 at a dilution of 1:6400 (approx 37.5ng/ml). Typical yields for anti-C4 Mab affinity purification procedures were:

Mab anti-Rodgers:1 (Anti-C4A) 150mls cell culture supernatant = **1.5mg**
Mab 1228 (anti-C4B) 1.0ml ascites = **4.0mg**
Mab T2. C5.12 1.5mls ascites = **4.84mg**

2.5 Affinity purification of R 340 Rabbit polyclonal anti-mouse IgG

Purification of this antibody was achieved by acid elution from a mouse IgG-conjugated Sepharose C4B column loaded in alkaline, high salt conditions.

Conjugation of Mouse IgG to Sepharose C4B

Materials

Cyanogen Bromide activated Sepharose C4B (600μg) (Pharmacia)
Mouse IgG, 7.5mg/ml (courtesy of Dr M.J Walport)
2ml syringe barrel and glass wool baffle
Amicon ultrafiltration apparatus and pressurised Nitrogen gas cylinder

Buffers

Coupling buffer: $NaHCO_3$ 100mM, NaCl 500mM adjusted to pH 8.3 with 5.0M NaOH
Blocking buffer: TRIS HCL pH 8.2
Washing buffer: NaAcetate 100mM, NaCl 0.5M adjusted to pH 4.0 with 5.0M NAOH
PBS, NaN_3 10mM, pH 7.2

Method

5.0mg protein/ml of sepharose C4B is recommended as the optimal ligand concentration [201]. The following procedure was therefore followed:

1. 600µg Sepharose C4B dry powder was added to 20mls of 1mM HCL for 20 minutes prior to washing with a further 200mls of the same solution in through a glass sinter filter under suction.
2. This was then washed with 5mls of coupling buffer, removed from the filter paper immediately as a wet gel and transferred with washing into a 5ml stoppered plain glass tube containing 3.25 mg mouse IgG in 2mls of coupling buffer.
3. The mixture was turned end on end for 2hrs at room temperature and allowed to settle for 30 minutes.
4. The supernatant was removed and the OD_{280} measured to estimate coupling efficiency **(87%)**.
5. The coupled gel was blocked by turning end to end for 2hrs at room temperature in 4mls of blocking buffer.
6. The blocked gel was then washed alternately in 1mM HCl and Acetate washing buffer by filtration under suction prior to washing with PBS, 10mM NaN_3, pH 7.2 prior to packing into the column with brief packing by high buffer flow rates to form an affinity column of **1ml bed volume at 3.0mg/ml Mouse IgG.**

2.6 Affinity purification of R 340 rabbit anti-mouse IgG

Materials

R 340 Rabbit anti-mouse IgG, 36mg/ml (courtesy of Dr M. J. Walport)
Mouse IgG-coupled Sepharose C4B affinity column as above

Buffers

Washing buffer:	TRIS HCL 1M, pH 8.3
Loading buffer:	TRIS HCl 1M, NACl 140mM, pH 8.3
Elution buffer:	TRIS HCl 1M, NACl 500mM, pH 8.3

Method

1. The affinity column was washed extensively with washing buffer then sample equilibrated with loading buffer.
2. The sample was mixed with an equal volume of 2x loading buffer and loaded twice under gravity and for 2 hrs thereafter by peristaltic pumping.
3. Elution was then undertaken with the high salt elution buffer, 1ml fractions collected and OD_{280} measured.

4. The presence of murine IgG binding activity in elution fractions was confirmed by Ouchterlony gel immunoprecipitation using Mouse IgG (as above) as antigen and the IgM containing peak pooled and dialysed against 6 ltrs of PBS, 10mM NaN_3 at 4°C for 24 hrs.
5. The resulting pool was concentrated by ultrafiltration in an Amikon filter at 20lbs/inch2 of Nitrogen prior to final estimation of OD_{280}.

Result

The OD_{280} elution profile of R 340 from mouse IgG affinity chromatography is shown below in figure 12. The yield was **1.5mg**.

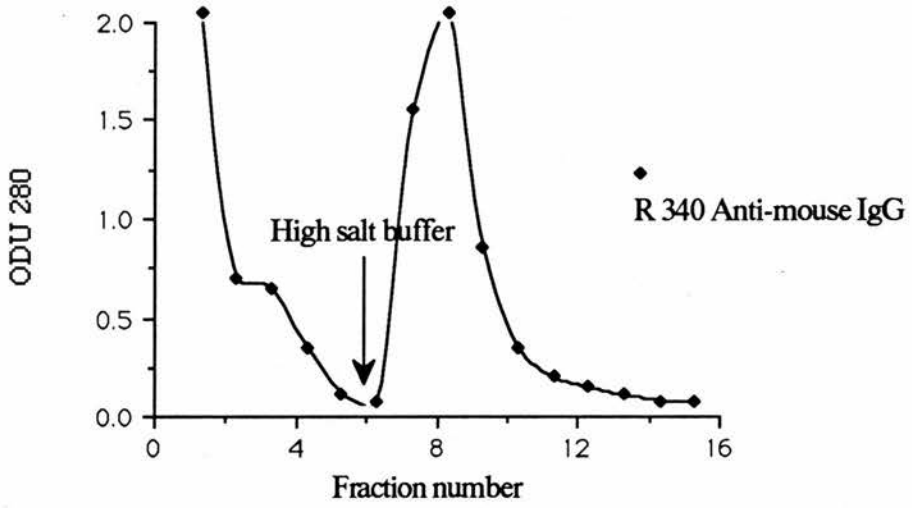

Figure 12. Affinity purification of R 340 rabbit anti-mouse IgG from mouse IgG-conjugated Sepharose C4B.

2.7 Affinity purification of Sheep polyclonal anti-C4

This procedure involved salt and acid elution of commercial polyclonal anti-C4 from a human C4-conjugated Sepharose C4B affinity column. The method and results are very similar to the foregoing but acid elution was required to release specifically bound material.

Materials

Affinity column: Human C4-conjugated Sepharose C4B, 2.5ml bed volume was kindly donated by Dr R. Harrison, MITI, Cambridge.

Anti-C4: Sheep anti-human C4 was obtained from Serotec (Serotec, Station Rd, Blackthorn, Bicester, Oxon).

Peristaltic pumps and Amicon ultracentrifugation apparatus were used as before

Buffers and eluates

Loading buffer:	TRIS 1m, NaCL 0.14M, ph 8.3
Salt elution buffer	TRIS 1m, NaCL 0.5M, ph 8.3
Acid elution:	Glycine 100mM, pH 2.3

Method

1. 5ml of antibody was equilibrated with 5mls of 2 x loading buffer.
2. After equilibration with buffer, the column was loaded with antibody by continuous cyclical perfusion using a peristaltic pump at 5mls/hr for 24hrs at 4°C.
3. The column was then eluted with salt elution buffer to remove weakly bound material and washed until the eluate OD_{280} returned to baseline.
4. Antibody was then eluted with glycine 100mM, pH 2.3 and 1ml fractions collected into test tubes containing an equal volume of washing buffer to partially neutralise the eluate.
5. Pooled fractions were immediately dialysed against 3 ltrs of PBS, 10mM NaN3, pH 7.3 for 24 hrs at 4°C to reduce acid damage.

Result

A broad elution peak was obtained and 8 1 ml fractions were pooled for dialysis and concentration. The final yield was **6.9mg**.

2.8 Purification of BH1 human hybridoma monoclonal aCL

BH1 is a human hybridoma IgM antibody obtained from the peripheral blood lymphocytes of a patient with the primary antiphospholipid syndrome. This antibody was purified by euglobulin precipitation followed by gel chromatography on Sephacryl S-300.

Materials

A 2.5x150 cms pre-packed gel filtration column (Bio-Rad Laboratories, Caxton Way, Watford, Herts) containing Sephacryl S-300 (Pharmacia) was kindly provided by Dr C. Winearls, Renal Unit, RPMS.

A variable rate peristaltic pump and tubing

Amicon ultrafiltration apparatus and pressurised Nitrogen gas cylinder

Sorval RC5B refrigerated ultracentrifuge and UB4 rotor
(Du Pont Co UK, Wedgewood Way, Stevenage, Herts)

Buffers

Euglobulin precipitation buffer: 2mM Phosphate buffer, pH 6.0

Gel filtration buffer: 10mM TRIS HCL, 150mM NaCL, pH 7.3, Conductivity 15.7mho at 4°C. Buffer was degassed at 4°C prior to use.

Method

Euglobulin precipitation

1. 500mls of cell culture supernatant was concentrated 10 fold by ultrafiltration under 50lbs/inch2 pressure using an Amicon filter and pressurised Nitrogen cylinder.
2. This was dialysed against 6 ltrs of euglobulin precipitation buffer at 4°C for 24 hrs.
3. The precipitate was harvested and washed in 3 changes of buffer by centrifugation at 2500rpm and redissolved in 1ml running buffer.

Gel filtration: all procedures were carried out at 4°C in a cold room

1. The column was equilibrated until the conductivity equalled running buffer and the resolving capacity of the gel estimated by filtration of a mixture of known human IgG and IgM. Human IgM appeared between 40 and 64 mls elution volume, while IgG was eluted between 80 and 100mls.
2. The redissolved euglobulin precipitate was then carefully loaded onto the top of the column by injection via a 3-way tap and the column pumped at 20mls/hr. All air was excluded from the column.
3. 4ml fractions were collected and the OD_{280} measured.
4. Column fractions were assayed for IgG and IgM by Ouchterlony immunodiffusion. IgG was not found while IgM bearing fractions were pooled and dialysed against 3 ltrs of PBS, NaN3 10mM prior to final concentration and estimation of protein concentration.

Results

The elution profile for Sephacryl S-300 gel filtration of euglobulin precipitate of human monoclonal aCL (anti-negatively charged phospholipid) BH1 is shown below (figure 13). The elution profile of test human IgG is also shown for comparison, though not to the same scale. While there was a suggestive peak at the IgG position on the BH1 elution, no IgG was detected by gel immunoprecipitation. The final yield from this procedure was **6.0mg of IgM.**

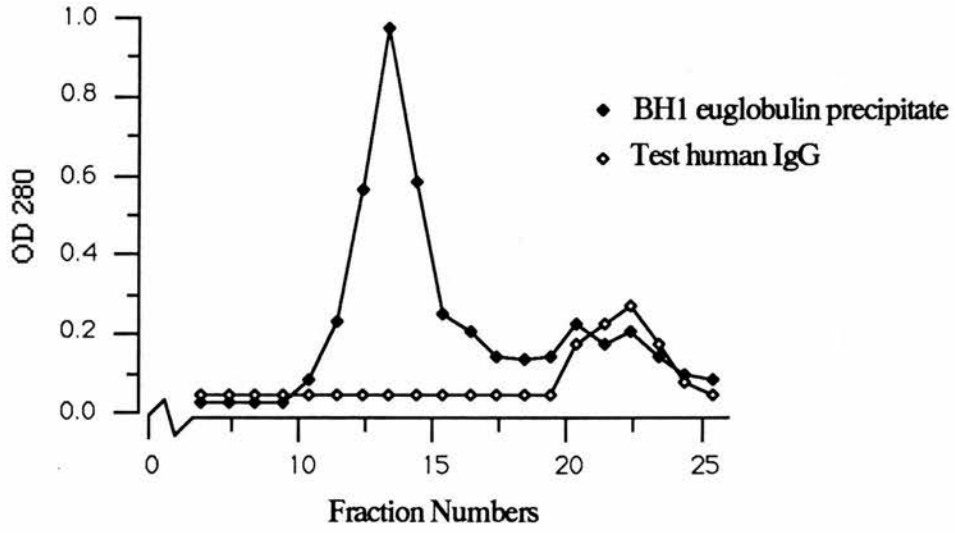

Figure 13. Gel filtration of Mab BH1 anti-phospholipid antibody euglobulin precipitate.

2.9 Radiolabelling Procedure for Monoclonal Antibodies

Radiolabelling was carried out by the "Iodogen" method [202]. In general, 1-2.5μCi of fresh $Na^{125}I$ (Amersham International Ltd, Lincoln Place, Greenend, Aylesbury, Bucks) was used to radiolabel 100μg of antibody and all procedures were carried out in a fume cupboard in a designated "hot" laboratory. Latex gloves were worn at all times when handling radioactive materials and all surfaces monitored by hand held Geiger-counter after procedures.

Materials

1.5ml conical, capped polypropylene tubes (Eppendorf Gerateban, Hamburg 63, W Germany) coated with Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) by evaporation from a volume of 1ml dichloromethane.

$Na^{125}I$ of known specific activity (Amersham)

Sephadex-G25 "PD 10" Gel filtration column (Pharmacia)

Buffers

PBS and PBS, 1% bovine serum albumin (BSA)

Method

1. One μl of $Na^{125}I$ at 1mCi/ml was added to 100μg of antibody in PBS/10mM NaN_3 (100μl approximate volume) and agitated for 15 minutes at room temperature.
2. A 2.5μl aliqout of this reaction mixture (pre-separation) was retained.
3. A pre-packed PD10 (Pharmacia) column (10 ml bed volume) was blocked with PBS

1% BSA, washed with 50mls of PBS and the top just allowed to dry by gravity elution of the buffer.

4. The bulk of the reaction mixture was then carefully pipetted, with a small volume of washing, onto the centre of the column bed and eluted with 500µl aliquots of PBS, allowing each aliquot to enter the gel prior to addition of the next.

5. The activity of each fraction was monitored and the peak of protein bound radioactivity was routinely found in the 5th and 6th of these 500µl fractions which were then pooled, free radioiodine appearing in subsequent fractions was discarded.

6. A 2.5µl aliquot of pooled labelled antibody (post-separation) was also retained prior to addition of 0.1ml 30% BSA solution (approximately 3% final concentration) as stabilising protein.

The efficiency of radiolabelling and the specific activity of labelled antibody was then calculated by precipitation of all protein in the pre- and post-separation aliquots with 10% (w/v) Tri-Carboxylic Acid (TCA) in the following manner

1. Fifty µl of normal serum was added to each retained (pre- and post-labelling) 2.5µl aliquot to act as excess (carrier) protein followed by 500µl TCA and the precipitated protein separated by centrifugation at 10,000 G.

2 In each case, a 100µl aliquot of supernatant (S) was removed and counted separately from the remainder containing the protein pellet (P). The protein-bound counts are thus given by subtracting (S x 5.5) from the total counts (S + P).

3. The percentage of protein-bound counts in the pre-separation specimen indicates the efficiency of radio-iodine incorporation and was routinely > 90%.

4. Since the concentration of protein (X) and the radioactivity in the 2.5µl pre-separation specimen are known, the concentration in the post-separation specimen (Y) and the specific activity (cpm/µg antibody) may be calculated in the following manner.

$$\frac{\text{cpm (P) pre-separation x 400}}{\text{X µg/ml}} = \text{cpm/µg (specific activity)}$$

$$\frac{\text{cpm (P) post-separation x 400}}{\text{cpm/µg (specific activity)}} = \text{Y µg/ml (of labelled antibody)}$$

Specific activities of **1-2 x 10^6 cpm/µg** and recovery of **60-80%** of labelled protein were routinely achieved and this method was used unless otherwise stated.

2.10 Radioligand enumeration of cell surface antigens

These assays were performed by application of the method previously described [175].

Preparation of test erythrocytes

Buffer: PBS containing 1% bovine serum albumin (BSA), 10 mM NaN_3 (PBS/BSA) was used throughout

Washing erythrocytes

In all experiments, unless otherwise stated, erythrocytes were separated, washed and packed by centrifugation at 2500 rpm in an MSE "Mistral" 3000 centrifuge (MSE Ltd, Scientific Instruments, Bishop Meadow Rd, Loughborough, Leics.) and all blood samples were processed within 4 hrs of venesection. After packing, plasma and buffy coat cells were removed by suction aspiration and the erythrocytes washed 3 times in 5 volumes of cold (4°C) PBS/BSA buffer by centrifugation and aspiration.

Cell counting

Packed cells were then resuspended in an equal volume of cold PBS/BSA buffer and counted by a semi-automated technique. Cells were first diluted to 1/20,000 in "Isoton" (Coulter Electronics, Northwell Drive, Luton, Beds) routine haematological diluent in two steps using a Dual Dilutor II (Coulter) automated dilutor. Erythrocyte concentration was then measured by using a mark II semi-automated "Coulter"-counter (Coulter) after adjustment of the aperture current to the appropriate setting for the measurement of red cells. Routine automated haematological analysis was performed on 10 samples by the department of haematology, Hammersmith Hospital, to exclude the presence of excessive numbers of white blood cells and to calibrate the Coulter-counter used. A mean of 1.87×10^9 white blood cells/l and 15.57×10^9 platelets/l were present after washing and linear regression analysis of measurement by Coulter-counter and by the routine haematology laboratory gave a slope of 1.05 and a regression coefficient of 0.982. Duplicate analysis of 20 specimens gave a coefficient of variation for the measurement of erythrocyte concentration of 2.75%. Washed erythrocytes were then adjusted to a concentration of 5.0×10^{12}/ml in PBS/BSA buffer and stored at 4°C prior to analysis. Initial observations showed that ligand numbers were stable for up to 1 week under these conditions, thereafter, haemolysis occured.

Radioligand binding assays

Materials

1 ml LP3 test tubes (Elkay Lab Products Ltd, Unit 5, The Ridgeway Centre, Basingstoke)

250µl polypropylene microfuge tubes (Elkay)

Dibutylpthalate and dinonylpthalate (BDH)

Benchtop ultracentrifuge (Beckman Instruments Ltd, Sands Industrial Estate, High Wycombe, Bucks)

Gamma counter. LKB "1260 multigamma" gamma counter and printer (LKB-Pharmacia, Midsummer Boulevard, Milton Keynes)

Method

1. Aliquots of radiolabelled antibodies of known specific activity were adjusted to a concentration of 30µg/ml (the concentration of antibody prior to admixture with erythrocytes will be referred to as the initial concentration) in PBS/BSA buffer.
2. Erythrocytes were adjusted to $5.0x10^8$/ml buffer and a 75µl aliquot of antibody was added to 375µl erythrocytes at in a Beckman LP3 polypropylene test tube, capped and incubated for 30 minutes in a 37°C water bath with regular agitation. The reaction concentrations of antibody and cells were therefore 5µg/ml and $3.33x10^8$/ml respectively.
3. The reaction was then stopped by cooling the tubes in a melting ice water bath.
4. Duplicate 150µl aliquots of reaction mixture ($5x10^7$ cells) were then carefully layered onto 75µl aliquots of a mixture of 4:1 dibutylpthalate and dinonylpthalate in a 250µl polypropylene microfuge tube and bound counts (pellet) separated from free (supernatant) by ultracentrifugation at 10,000G for 5 minutes in a bench-top ultracentrifuge.
5. After freezing at -70°C for 1 hr, unbound label in the aqueous phase was separated from bound counts in the erythrocyte pellet by cutting the polypropylene ultracentrifuge tube through the solidified oil layer with wire cutters. The radioactive disintegrations of duplicate pellet and supernatants were then measured and expressed as mean counts per minute (cpm).

Correction for background binding

A certain proportion of the radioactive counts in the erythrocyte pellet (the background) are carried there by non-specific trapping amongst the cells during centrifugation. An initial approach to correction for non-specific background binding was to perform each incubation in the presence of 250% excess unlabelled (cold) blocking antibody. However, this blocking concentration proved to be inadequate and the mean binding of 5 monoclonal

antibodies (anti-C4A, C4B, C4d, C3d, C3dg) in the presence of competing cold antibody was 12.5%. There were insufficient reagents to pursue this method of background correction further with higher cold antibody concentrations in each reaction. I therefore analysed the percentage of radiolabelled antibody appearing in the erythrocyte pellet in reactions where no specific binding was expected.

Method

A total of 12 assays were performed under standard conditions with antibodies and cellular substrates where no specific binding was expected.

1. Anti-C4A was incubated with erythrocytes from a C4A deficient donor.
2. Anti-C4B was incubated with erythrocytes from a C4B deficient donor
3. Mab clone 4 [198] was incubated with normal erythrocytes. (Clone 4 recognises C3c which is released from the erythrocyte surface in the presence of Factor I and CR1 in whole blood, and is therefore a negative control antibody). Assays were conducted in duplicate.

Result

The mean percentage non specific binding in the erythrocyte pellet in these experiments was **0.217%** of the **total** cpm offered in the reaction and this figure was used for background correction in radioligand binding studies. I also noted that antibody background rose with the "age" of the label, *ie* the time since the radiolabelling reaction,.and therefore adopted the procedure of replacing all labelled antibodies with a fresh aliquot after a period of 45 days and omitted all measurements made with older labels from further analysis.

2.11 Standardisation for variation in specific activity between aliquots of radiolabelled antibody

Alterations in the radiolabelling reaction conditions, notably in the duration of the iodogenation reaction or the quantity of Na ^{125}I added may lead to variation in the specific activity of the radiolabelled monoclonal probe and therefore of results obtained from successive labelled aliquots of the same antibody. To standardise for this effect, duplicate measurements of at least 6 samples were made with "old" and "new" labels as each successive labelled aliquot of each antibody was introduced. The results were compared by linear regression, plotting results obtained with the "old" label on the Y axis. The slope of the regression thus represents a cumulative correction factor and was used to standardise the quantification of antigens to the equivalent measurement by the first labeled antibody preparation. This method does not improve the "veracity" of the quantification since the first labelled preparation may not be optimal. In practice, the majority of labelling reactions were quite closely equivalent and the cumulative correction factor applied remained near unity, with the exception of one labelling of anti-C4A and the second labelling of anti-DAF.

2.12 Calculation of ligand binding sites per erythrocyte

Mean pellet counts were corrected for non-specific binding, as described above, to give erythrocyte-bound cpm. After labelling and calculation of the labelled antibody concentration, triplicate 100ng aliquots of antibody were placed in separate LP3 test tubes and counted. The median sample was preserved by covering with melted candle wax and used as a standard to correct for decay of the label activity with time. Given the number of cells in the pellet (150µl at 3.33×10^8/ml: 5×10^7 cells), the number of molecules in 100ng (4×10^{11}), it is possible to calculate the number of antibody molecules bound/cell using the formula:

$$\frac{\text{cpm-bound/reaction}}{\text{cpm 100ng}} \times \frac{4.0 \times 10^{11}}{5.0 \times 10^7}$$

This can be simplified to:

$$\frac{\text{cpm-bound/reaction}}{\text{cpm 100ng}} \times 8000$$

The number of molecules/cell calculated in this way was then standardised for differences in labelled antibody activity to give the final enumeration.

2.13 Low ionic strength method for coating erythrocytes with C4

Erythrocytes may be coated with C4 by incubation with serum at low ionic strength [203]. Incubation of packed erythrocytes and serum at concentrations of approximately 5 and 10% respectively in 10% sucrose solution produce a strong coating of covalently bound C4 on the cell surface. These conditions may be varied to produce the optimal density of cell coating.

Method

1. Type O erythrocytes from a healthy volunteer were washed 3 times in cold PBS/BSA buffer and packed by centrifugation as described above.
2. 20µl packed cells, 40µl donor serum (self) and 400µl 10% sucrose were incubated at 37°C for 30 minutes with regular agitation prior to washing in cold PBS/BSA buffer as described above.
3. Control cells incubated with serum, 10mM EDTA were prepared in the same fashion.
4. 200 mls of packed cells were incubated with radiolabelled anti-C4d and Protein A at approximately 250µg/ml (5×10^5 cpm) for 30 minutes at 37°C in a final volume of 0.5ml PBS prior to separation of bound from free antibody by centrifugation of duplicate 50µl

aliquots through oil mixture as before.

5. Radioactivity bound to erythrocytes (pellet) and free label (supernatant) were then separated by centrifugation and the erythrocyte-bound cpm measured as before.

Result

Binding of antibody expressed as % cpm offered is shown below in table 6 and demonstrates that C4 is deposited on erythrocytes by the classical pathway, in a calcium dependent manner. Protein A acts as a negative control and also demonstrates that deposition of autologous IgG does not occur.

C4d		Protein A	
Serum	Serum EDTA	Serum	Serum EDTA
55%	**0.83%**	**0.90%**	**0.57%**

Table 6. Calcium dependent deposition of C4d to human erythrocyte by incubation at low ionic strength.

While this experiment is semiquantitative (erythrocytes were not enumerated) experiments under standard radioligand binding assay conditions demonstrated deposition of up to 4 x 10^5 C4 molecules/cell.

2.14 Radioimmunometric assay of C4 isotypes in serum

A two armed immunoradiometric assay was devised for the measurement of serum C4 isotype concentrations. Briefly, C4 was captured from serum by polyclonal sheep anti-C4 immobilised in removable microtitre wells. Duplicate aliquots of each sample were incubated on each of three identical plates for the parallel estimation of C4A, C4B and C4d. After washing, captured C4 was probed with murine monoclonal antibody and bound antibody revealed with radiolabelled rabbit anti-mouse IgG. C4 bound was quantified by comparison with serial dilution of pool normal human serum and results calculated by computer spread-sheet specifically written for the purpose.

Materials

96 well removable microtitre wells, "Imulon 2 Removawells"
(Dynatech laboratories Ltd, Daux Rd, Billingshurst, Sussex)

Single chamber and multi-barrel "pipetteman" pipettes
(Gilson SA, BP No 45-95400, Villiers le Bel, France)

LKB "1260 multigamma" gamma counter and printer (LKB-Pharmacia)

Affinity purified: sheep polyclonal anti-C4, murine monoclonal anti-C4A, C4B and C4d, ^{125}I rabbit polyclonal anti-Mouse IgG (see above)

Buffers

Washing and blocking buffer: PBS, 0.2% BSA, 0.05% Tween 20, NaN_3 10mM, pH 7.4

Method

Washing and blocking buffer was used for incubation of samples, washing of unbound material and blocking of non-specific binding. 50µl aliquots were used at each step.

1. Polyclonal anti-C4 was bound to 96 well microtitre assay plates by incubation at 4°C for 18 hrs at 10µg/ml PBS, pH 7.4. Assay plates consisted of strips of separable wells located in 96-well holders and were easily removed for the final measurement of radioactivity bound in each individual well.
2. After washing and blocking of non-specific binding with 6 changes of buffer, duplicate 29.76nl aliquots of test serum (50 µl serum at 1:1680 dilution) was incubated on each of three identical plates for 18 hrs at 4°C. A standardised technique was employed to improve assay reproducibility. 10µl aliquots were obtained using a 5-20µl variable volume pipette, previously calibrated by weighing dispensed distilled water. Samples were diluted in 2 steps by the addition of 10µl neat serum serially to buffer. The pipette-tip was carefully wiped with tissue to remove adherent serum and was changed at each step. Each well was individually aspirated to dryness by suction between each change of washing buffer, taking care not to disturb the bound material in the base of the well.
3. Unbound material was removed by 6 washes of buffer, the plate covered with laboratory clingfilm to reduce evaporation and incubated with 2.0µg/ml of anti-C4A, C4B and C4d for 4hrs at 37°C
4. After washing and blocking as before, 100, 000 cpm/well (approximately 2µg/ml) of radiolabelled rabbit anti-mouse IgG was incubated for 4hr at 37°C and the plate finally washed and dried prior to separation of the wells and individual measurement of bound radioactivity in the gamma-counter. The mean disintegrations of ^{125}I-labelled anti-mouse IgG specifically bound in each duplicate pair of wells are expressed as counts per minute (cpm).

Standard samples, calculation of results and assay variability

Pool serum was donated by 20 normal volunteer laboratory and hospital staff (10 female and 10 male), median age 23.5yrs. Serum was separated and stored in aliquots at -70°C within 4hrs of venesection. Pool serum was serially diluted 8-fold from 1:100 in blocking buffer and 50µl aliquots incubated in duplicate in the same manner as test samples.

Standard samples were incubated on each assay plate. Sera from healthy donors deficient in C4A, C4B and C4d were included to provide a value for non-specific, background binding which was subtracted from each test result. Two further normal sera expressing both C4 isotypes were included in each assay as standard samples to allow inter-assay comparisons.

Calculation of results

The assay was linear in the range 125 - 15.6nl of serum added, and allowed quantification in the range 250 - 7.8nl serum added. Typical assay standard curves are shown below in figure 14, along with the dilution of test sera (1: 1680, 29.76nl added).

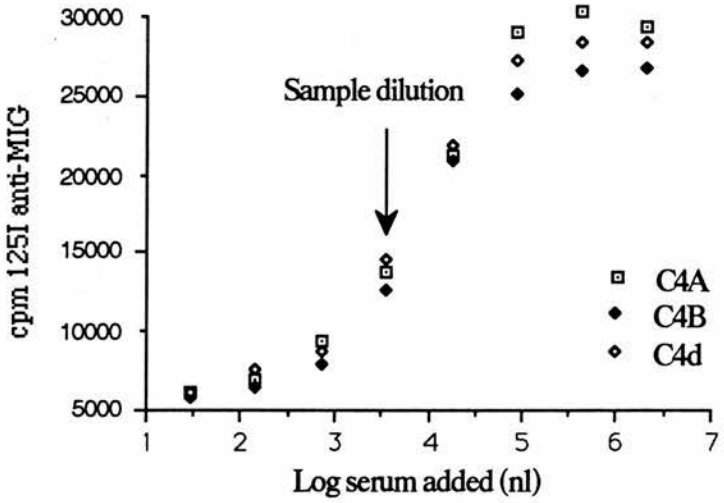

Figure 14. Standard curves of dilutions of pool serum measured as C4A, C4B and C4d for the quantification of serum C4 levels. The dilution of test samples is indicated by the arrow.

Assay results were calculated with a Macintosh, SE 20 desk-top computer (Apple Computers Inc) utilising an Excel 1.53 spreadsheet programme (Microsoft Software Ltd) written by me for the purpose. The mean cpm of each standard dilution was calculated and corrected for background binding in the presence of C4 deficient serum as above. A linearised standard curve was then constructed in the following manner. CPM associated with each dilution of pool serum was expressed as the percentage of the maximum binding associated with the top dilution of pool serum (1:100) and transformed by the equation

$$\text{Logn}(100-\%\text{max})/\%\text{max}$$

This is equivalent to the logit transformation [204] (Ln p/(1-p)) and improves responsiveness of the assay at the extremes (the sigmoid portion of the response curves) extending the range of values which can easily be mathematically modelled. In practice this improved the measurement of values between 250 - 125nl of serum added (dilutions 2 and 3 of pool serum) and 15.625 - 7.81nl of serum added (dilutions 6 and 7). These values

were then plotted on the X axis and regressed against the log of the quantity of serum added in nanolitres using Cricket Graph 1.31 software (Cricket Software). This programme allows a number of line models to be constructed and I found that a second order polynomial expression ($Y= A+Bx+Cx^2$) fitted the data most closely, frequently with a correlation coefficient of unity. The regression coefficients were then be used in the spreadsheet described above to calculate the equivalent quantity of pool serum (nl pool serum) represented by the mean cpm binding from the incubation of standard dilutions of test serum. To illustrate the accuracy of this method, figure 15 shows the same standard curves as in figure 14 recalculated as nl of serum from the experimentally measured cpm at each point, with the actual quantity of serum in each dilution illustrated and joined by the line.

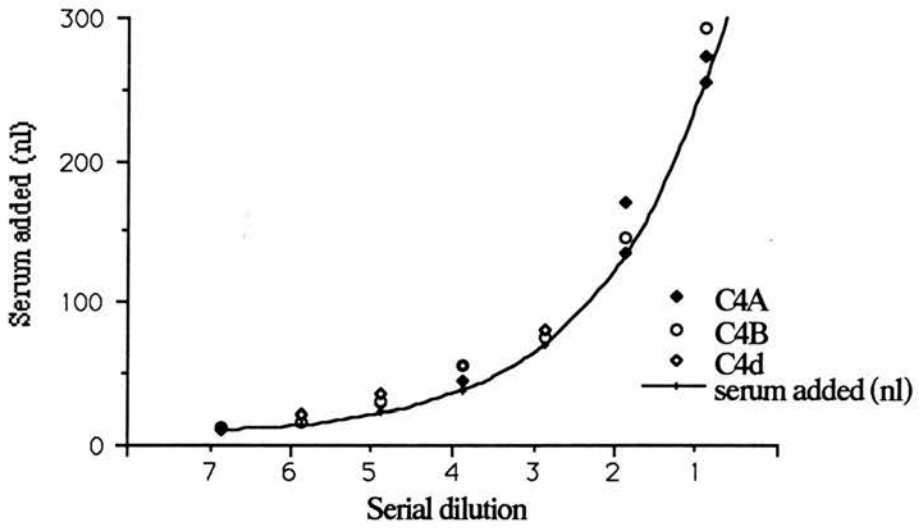

Figure 15. Data for the same standard curves as in figure 14 treated as unknown samples and computed as nl of serum, with the actual quantity in each dilution shown for comparison.

Calibration of C4 assay

Having calculated the quantity of C4 in test samples as the equivalent volume of pool serum (nl pool serum), results were initially calculated as the **percentage** of the volume at test dilution (50µl/1680 = 29.76nl) and expressed as **% pool normal human serum (% pool serum)**. These values were then calibrated by comparison with total C4 in mg/l measured in 11 test samples (including the assay standards) by laser nephelometry. Nephelometry was kindly performed by Dr B P Morgan, Department of Chemical Pathology, University Hospital of Wales, Cardiff. Figure 16, overleaf shows that the regression of C4d measured by RIA (expressed as % pool serum) with C4 total measured by nephelometry (also expressed as % of the C4 in mg/l in samples of pool serum assayed by nephelometry) approaches a slope of 1.0, implying that there is no systematic variation

in either measurement. Figure 17.below demonstrates the calibration of the assay by linear regression of C4d (RIA, % pool serum) with nephelometric C4 total expressed as mg/l. The slope of regression is 2.4 Values of C4d (% pool serum) were therefore adjusted by 2.4, while values of C4A and C4B were adjusted by 1.25, on the assumption that pool serum contains equal quantities of both isotypes.

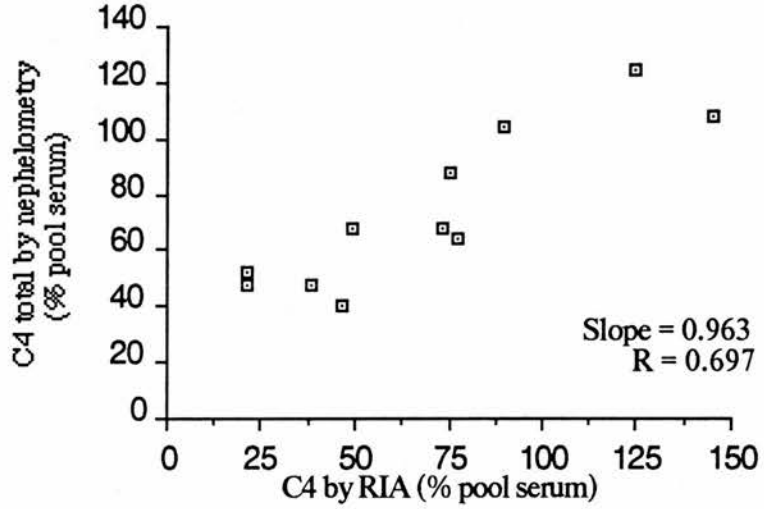

Figure 16. Correlation of C4 total measured by RAI with C4 measured by nephelometry. Both measurements are expressed as % pool serum.

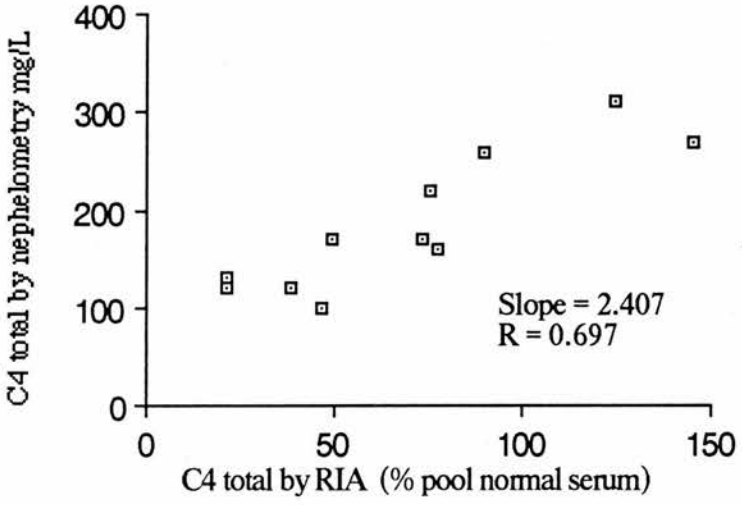

Figure 17. Calibration of C4 RIA in mg/l.

Inter-assay variability

The 4 standard samples (C4AQ0, C4BQ0, normal 1 and normal 2) were measured in each assay. Eight sets of assays were performed to measure C4 in patients with SLE (for comparison with erythrocyte and HAGG C4 deposition) and in genotyped normal sera for the analysis of C4 genotype and phenotype relationships. The mean and SD of the 8 measurements of C4A, C4B and C4d available for each of these 4 samples (excluding the deficient alleles) was taken and the the coefficient of variability found. The average value of these coefficients, the inter-assay variability of C4 measurements was **13.74%.**

Intra-assay variability

In the analysis of C4 genotype and phenotype, a total of 45 measurements amongst 16 individuals were made in duplicate in the same assay run (for the purposes of this analysis the measurement of C4A, C4B and C4d in each sample are considered to be independent variables and referred to as measurements of C4). Figure 18 shows the regression analysis of the first and second measurements of these samples, the correlation coefficient (R) is 0.70 and regression coefficient is 0.963.

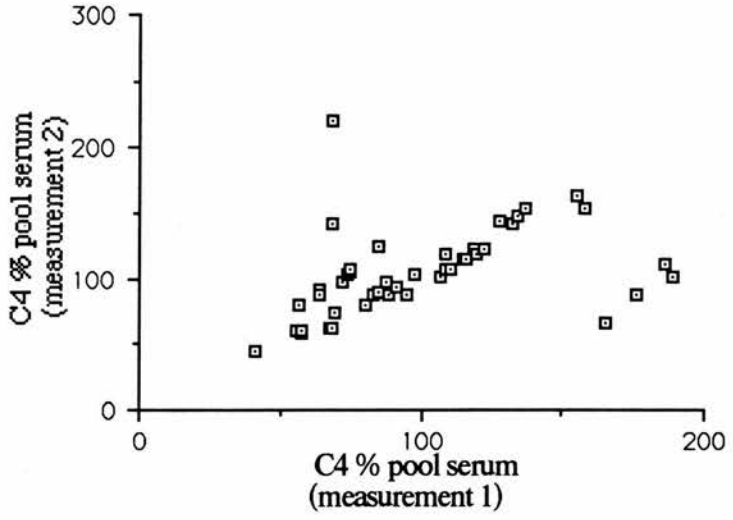

Figure 18. Regression analysis of duplicate measurements of C4 in serum. The correlation coefficient (R) is 0.70 and regression coefficient is 0.963.

This method of assessing variability between two measurement has been criticised [205], and figure 19 (overleaf) shows the result of the analysis suggested by these authors for the repeatability of clinical measurements. Briefly, the differences between the 1st and 2nd recordings are plotted against the average of the 2 measurements. The mean (-0.323) and the standard deviation (SD) of the differences (39.47) is then calculated. To comply with

the British Standards Institution definition of acceptable repeatability (the repeatability coefficient) 95% of differences should lie within 2 SDs of their mean. For this assay, **88.9%** of differences lie within this range.

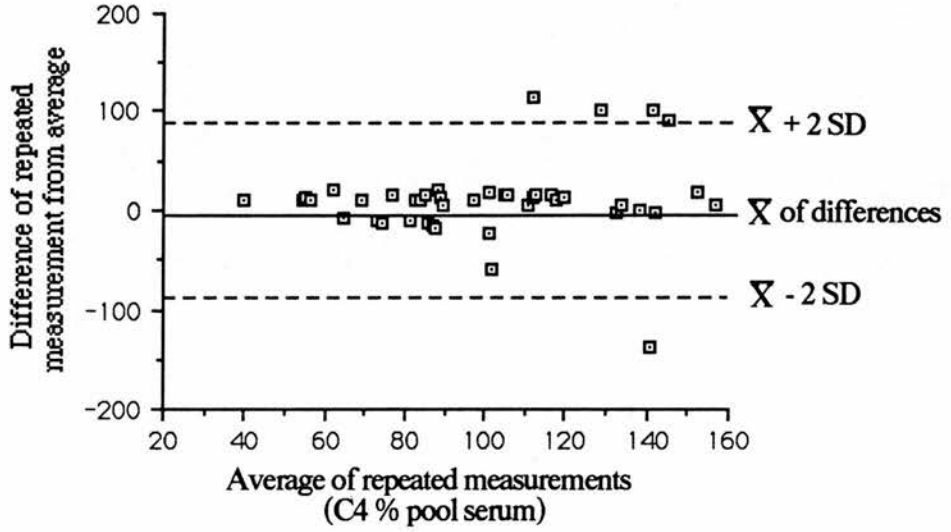

Figure 19. Plot of the differences of repeated C4 measurements from the average of the 2 values (Y) and the average of the measurements (X). The mean of the differences (— X) and mean plus or minus 2 standard deviations (-- X+2SD, X-2SD) are also shown.

The mean of these differences expressed as a percentage of the average of the measurement, the coefficient of variation, is **19.9%**. However, as may be seen from figures 18 and 19, a small number of measurements (7) appear to show extreme variability and the median percentage intra-assay coefficient of variation is **7.0%**.

2.15 Quantification of immune complex C4 deposition by ELISA

A modification of the serum C4 RIA was developed to quantify the deposition of C4 isotypes on test immune complexes in vitro. In view of the importance of DNA-antiDNA ICs in the pathogenesis of SLE, attempts were made to quantify C4 deposition in this IC system. Briefly, purified IgG with high DNA binding activity (measured by Farr assay) was prepared from SLE serum by saturated ammonium sulphate precipitation and ion exchange chromatography using Sephadex DE52 gel. DNA of known molecular weight was purified from salmon sperm DNA by phenol/chloroform extraction, Hind III DNAse digestion, gel electrophoresis and electro-elution from gel fragments. This was radiolabelled with ^{32}P CTP by the Klenow fragment end filling method. Immune complexes were prepared by incubation with purified anti-DNA using serum, EDTA and DNAse digested DNA complexes as controls. Despite employing a number of solid phase ligands and probes (including protein A, polyclonal and monoclonal anti-C4, monoclonal anti-C3d, C3dg, C3c and C3 neoantigen) it was not possible to produce a quantitative

assay system. This was in part due to difficulties in reducing background, non-specific binding of DNA. Successful quantification was achieved using heat aggregated IgG (HAGG) as the test immune complex.

Preparation of HAGG

Heat aggregates were prepared by the internationally standardised method [206].

1. "Sandoglobulin" sterile human IgG was obtained from Sandoz Pharmaceuticals (Frimley, Surrey), dialysed extensively with PBS and stored at -70°C without preservatives (Sodium Azide inactivates the peroxidase colour reaction).
2. Stock IgG was adjusted to 20mg/ml PBS buffer and aggregated by heating at 63°C (internal temperature of test tube) for 30 minutes in a thermostatically controlled water bath.
3. After aggregation, the reagent was centrifuged at 10,000 G for 30 minutes and subaliquoted for storage at -70°C prior to use.

Elisa assay

Buffers

Washing buffer: PBS, Tween 20 0.05%

Blocking buffer: PBS, 0.05% Tween 20, 0.2% BSA, 10mM EDTA

Complement Fixation test diluent (CFD), (Oxoid)

Method

50µl aliquots of all reagents were used and unbound reagents were removed with 6 changes of washing buffer and non-specific binding blocked by incubation with blocking buffer for 1hr at 37°C between each step.

1. Sterile human C1q was obtained (Sigma) and incubated in flat bottomed 96 well ELISA plates (Nunc A/S, DK-4000 Roskilde, Denmark) at 10µg/ml in PBS buffer pH7.4 for 18hrs at 4°C.
2. Test sera were stored at -70°C prior to use, thawed rapidly in a 37°C water bath and kept on ice during sample preparation. Stock 200mM EDTA was used to prepare a control aliquot (10mM EDTA final) of each sample.
3. HAGG was diluted 1:40 in CFD and a 10µl aliquot added to 90µl of test and EDTA control serum (final concentration **50µg HAGG/ml serum**) in a 2ml test tube and incubated in a water bath at 37°C for 30 minutes with frequent agitation.
4. The reaction was stopped by the addition of 400µl of iced blocking buffer, 10mM EDTA and duplicate aliquots pipetted onto each of 3 identical ELISA plates for the parallel assay of C4A, C4B and C4d. These were incubated at 37°C for 4hrs prior to washing and blocking as above.
5. Plates were then serially incubated with 2µg/ml purified monoclonal anti-C4A, anti-

C4B and anti-C4d in blocking buffer, followed by peroxidase-conjugated polyclonal sheep anti-mouse IgG (Dako Ltd, 22 The Arcade, The Octagon, High Wycombe, Bucks)

6. Bound peroxidase-conjugated antibody was revealed with 5mg OPD (o-phenylenediamine dihydrochloride, Sigma) in 50mls 100mM Citric acid buffer pH 3.0, activated by the addition of 20µl hydrogen peroxide. The reaction was stopped after 10 minutes at room temperature with H_2SO_4 1M and the optical density at 482nM read in an MR 710 automated ELISA plate reader (Dynatech).

Standard samples, the calculation of results and assay variability

Pool serum was derived from 20 normal volunteer laboratory and hospital staff (15 female and 5 male), average age 23.5yrs. Serum was separated and stored in aliquots at -70°C within 4hrs of venesection. Eight serial dilutions of HAGG in CFD were made from a starting concentration of 4mg/ml. Aliquots of each dilution (10µl) were added to 90µls of pool normal human serum and to pool serum, 10mM EDTA as control followed by incubation on each plate, in parallel with samples, as described above. In addition HAGG at 50µg/ml (equivalent to test samples) was incubated in C4A, C4B and C4d deficient serum (and EDTA controls) and added to each plate as standard and negative control samples. The reference range was thus 400 - 3.125µgHAGG/ml pool serum and the assay was linear in the range 400- approximately 6.25µgHAGG/ml pool serum. Typical standard curves are shown below in figure 20.

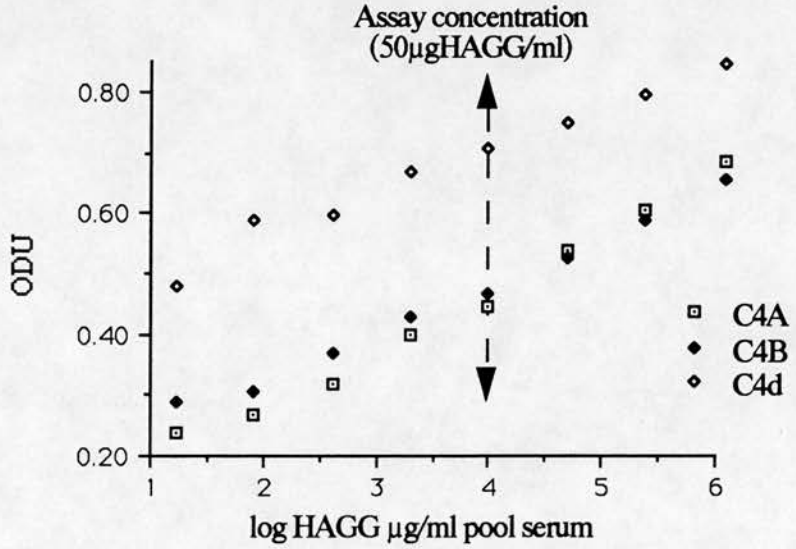

Figure 20. Standard curves for the quantification of C4A, C4B and C4d deposited on test immune complexes (HAGG) *in vitro*. Test samples are incubated with 50µg/ml HAGG as indicated. Mean ODU in unknown samples is converted to the quantity of HAGG producing equivalent binding in pool serum and then expressed as the % of 50µg/ml to give **% HAGG pool equivalent.**

Calculation of results

Test results were calculated by the same computer programme used for the C4 serum RIA from the 2nd order polynomial regression of the transformed mean ODU of the standard curves with the log concentration of HAGG in serum. The background non-specific binding (usually 0.17-0.22 ODU) relevant to each assay (eg C4A null serum, 10mM EDTA for C4A measurements) was subtracted from mean ODU readings in the calculation of specific C4 bound. The control value of background corrected binding in the presence of EDTA, which represents immune complexes, or other C1q binding material [206,207] in serum at the time of sample collection, was then subtracted from each measurement. For simplicity, the calculated quantity (μgHAGG equivalent/ml pool serum) was expressed as the **percentage** of the quantity of HAGG offered/reaction (50μg/ml serum), as above.

Intra-assay variability

Intra-assay variability was estimated by repeated assay of 12 test samples on the same assay plates, a total of 31 measurements. The mean of the differences between each measurement expressed as a percentage of the average of the 2 measurements, the coefficient of variation, is **13.75%**. The result of linear regression analysis of the first with the second of these measurements is shown below in figure 21. The slope of regression is 0.985 and the correlation coefficient is 0.976.

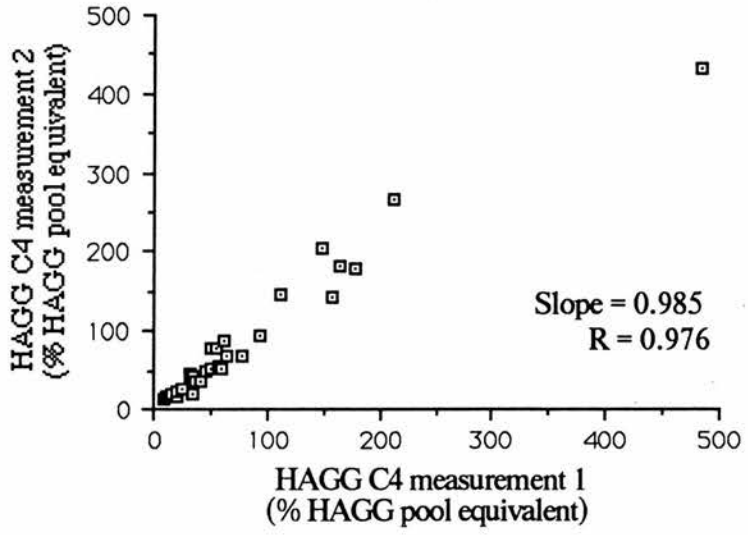

Figure 21. Linear regression analysis of the repeatability of the measurement of HAGG C4 deposition by ELISA assay amongst 31 measurements.

Variability has been analysed further by the method of Altman and Bland as described above. The mean of differences was -2.247 and the standard deviation of the differences

was 20.01. As explained, to comply with the British Standards Institution definition of acceptable repeatability (the repeatability coefficient) 95% of differences should lie within 2 standard deviations of their mean. For this assay , **90.33%** of the differences shown in figure 22 are within 2 SDs of the mean.

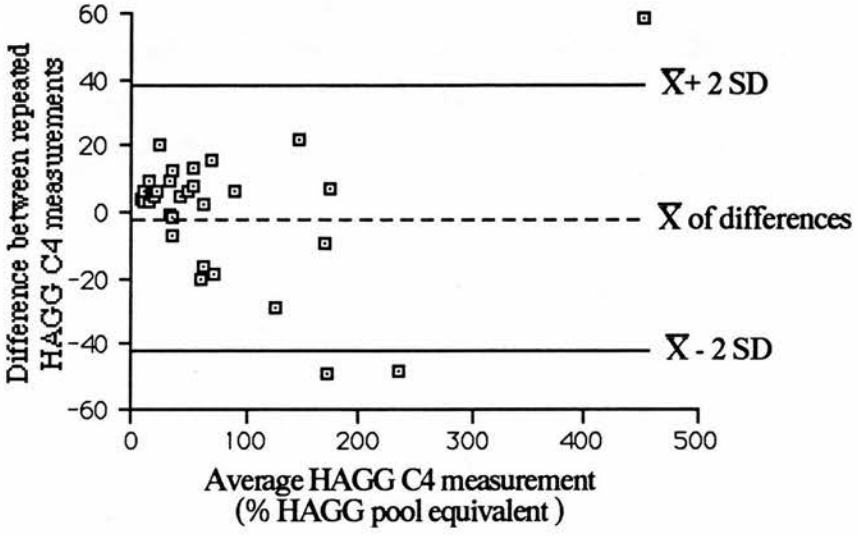

Figure 22. Repeatability of HAGG C4 measurements analysed by the method of Altman and Bland.

Estimation of intra-assay variability may be made by comparing the value of C4 total and C4A or C4B deposited on HAGG from standard null sera. Only six measurements were available for this comparison and the coefficient of variability between these recordings was **14.97%**.

Results

Chapter 3

Studies of the relationship between C4 genotype and phenotype

The extensive inherited polymorphisms of the two isotypes of C4, C4A and C4B, includes null alleles, associated with no expressed protein, and homoduplicated genes, encoding increased levels of C4 has been described in detail above. Identification of null alleles and homoduplications has posed considerable technical difficulties due to the great variation in C4 levels in normal subjects. Two advances have simplified this task. Firstly, the identification of DNA polymorphisms correlating with the presence of certain C4 null alleles, and, secondly, the availability of monoclonal antibodies which distinguish the two isotypic variants of C4. Nevertheless, even with the use of such monoclonal antibodies to assay separately C4A and C4B concentrations, it has been difficult to ascertain accurately genotype from phenotype in order to detect single C4 null alleles [97,209]. In addition, certain C4 alleles express the antigenic activity of the opposite isotype, a factor which may further obscure the relationship between phenotype and genotype.

The physiological significance of the extensive inherited polymorphism at the C4A and C4B loci is uncertain. The association of complement deficiencies and C4A null alleles with SLE has been described above. Many other associations between particular allotypic variants of C4 and diseases have been found, but these may be of no direct physiological significance and secondary to linked "disease susceptibility genes" encoded elsewhere in the major histocompatibility complex (MHC). For example, Felty's syndrome has been associated with the presence of C4B null alleles [210], though in this case C4B null alleles may be markers of particular haplotypes containing other, linked, disease susceptibility genes.

In order to study aspects of the relationship between C4 genotype and phenotype I have measured the serum concentrations of C4A and C4B and correlated these with C4 genotype amongst a cohort of normal relatives of patients with rheumatoid arthritis, who had participated in a family study of the major histocompatibility complex in this disease [211]. Results obtained confirmed extreme phenotypic overlap between different C4 genotypes. The factors contributing to this degree of overlap are not known but I have here studied the variation associated with gender, age and acute phase response, the influence of the presence of a null allele on the expression of associated genes and the effects of C4 alleles with abnormal antigenic reactivity.

Patients

Sera from 129 normal individuals were fully genotyped for HLA and complement genes as part of a family study of the HLA region in rheumatoid arthritis [211]. In order to ascertain the possible influence of gender on C4A and C4B expression, 32 haplo-identical sibling-pairs of opposite sex were identified from this cohort of subjects. Sera were stored in liquid nitrogen prior to use. C4 is an acute phase protein and I considered the possibility that family members of patients with RA might themselves have subclinical disease. I therefore measured levels of the acute phase reactant, C-reactive protein, in all samples and excluded from further analysis 1 sample from a subject with C-reactive protein concentrations of greater than 10mg/l. The acute phase response of C4d was studied amongst patients undergoing breast surgery and these are described further below.

Methods

Serum C4 isotype levels were measured by isotype specific RIA as described in general methods, chapter 2. Serum CRP was measured by Mancini radial immunodiffusion and I am grateful to Dr P.N. Hawkins, RPMS for the provision of purified CRP and polyclonal anti-CRP used for this assay.

Statistical analysis

Linear regression was used to measure the correlation between total C4 concentration and the sum of C4A and C4B concentrations. Otherwise non-parametric statistical tests were used throughout the study.

Results

3.1 Validation of C4 radioimmunoassay

The validity the assay was shown by the demonstration of a close correlation between total C4 given by the sum of the independent measurements of C4A and C4B with C4 total estimated as C4d. Linear regression analysis gives a correlation coefficient (R) of 0.917 and slope of 1.014 (figure 23). This compares with a recently reported ELISA immunoassay with a correlation of R = 0.85 [209].

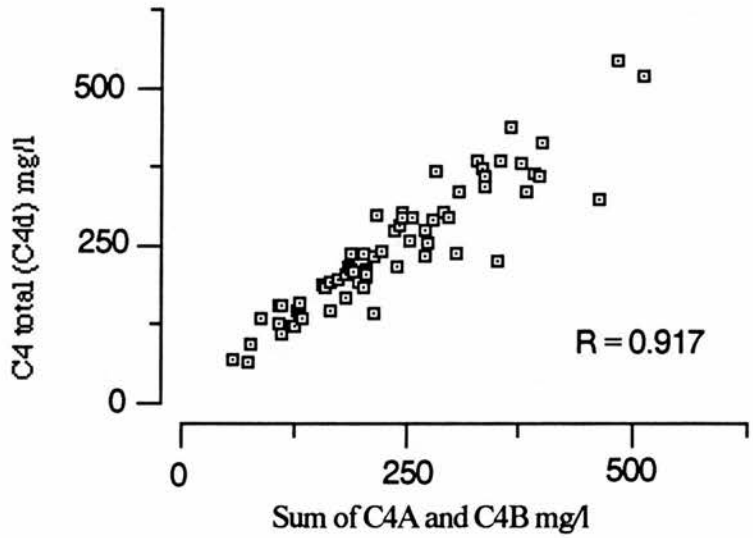

Figure 23. Correlation of total C4, measured as C4d, with the sum of the isotypes C4A and C4B measured individually.

3.2 Effect of number of C4 genes on serum C4 concentration

The levels of C4A found in individuals with 0 (homozygous C4AQ0), 1 (single C4AQ0), 2 or 3 (homoduplicated C4A allele) genes are shown in figure 24, below.

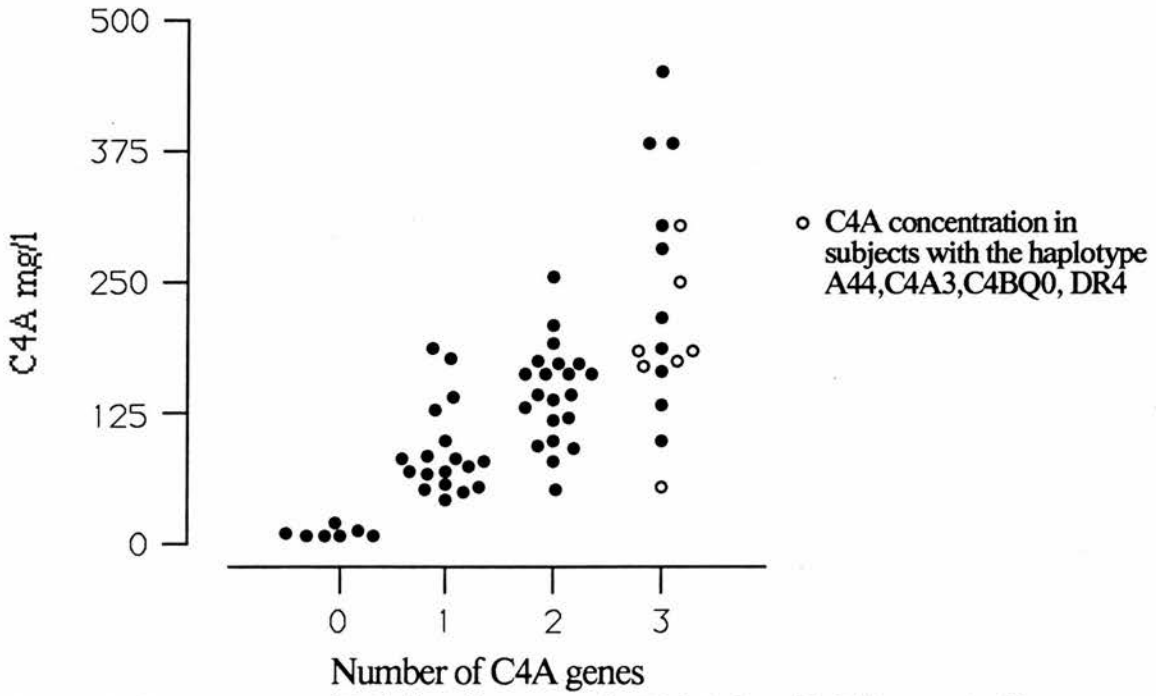

Figure 24. Concentration of C4A (mg/l) associated with 0,1, 2 and 3 C4A genes in 63 normal siblings of patients with rheumatoid arthritis. 7 subjects with the haplotype HLA- B44,C4A3, C4BQ0, DR4 are represented by unfilled circles o .

C4A was not detected in subjects with homozygous C4AQ0 alleles. The median concentration of C4A in subjects with 1 gene was 65.69mg/l (range 34.0 - 179.5mg/l; n=18), 2 C4A genes was 134.88mg/l (range 44.8 - 246.5mg/l; n=22) and 3 C4A genes was 241.12 mg/l (range 91.5 - 444.1mg/l; n=10). There were significant differences in C4A concentrations between the different groups of subjects (Kruskal-Wallis H=23.20, P<0.001), but a wide overlap in phenotypic expression of C4A concentrations was seen between the different genotypes.

The haplotype HLA-B44, C4A3, C4BQO, DR4 is associated with Felty's syndrome and has been demonstrated to contain two C4A alleles [210]. The expression of both of these genes was confirmed by the finding of C4A concentrations of 174.12mg/l (range 45.1 - 295.2mg/l) in 7 subjects bearing this haplotype. The concentration of C4B was 62.12mg/l (range 8.9 - 98.0mg/l) and of total C4, 237.5mg/l (range 65.5 - 356.5mg/l), in these 7 subjects (Fig 24). One individual was homozygous for this haplotype, *ie* possessed 4 C4A genes, and showed a C4A concentration of 339.12mg/l with absent C4B.

No duplicated C4B genes were present in our population. The ranges of C4B concentrations associated with 1 or 2 C4B genes is shown below in figure 25

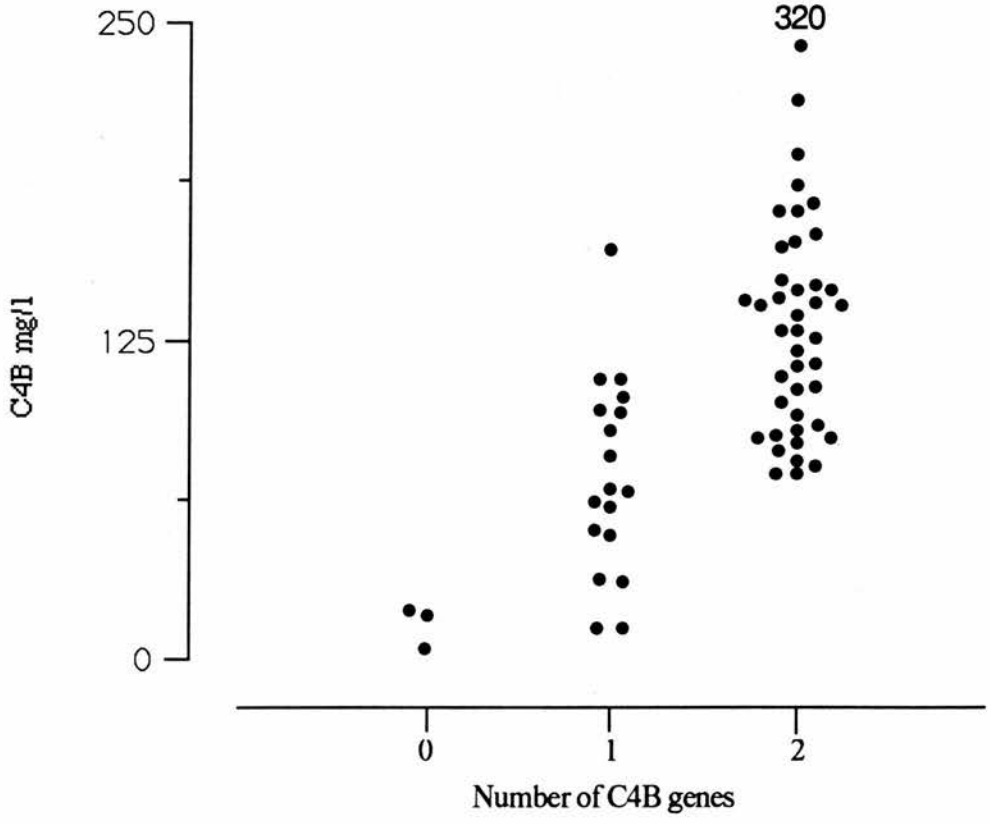

Figure 25. Concentration of C4B (mg/l) associated with 0 (homozygous deficiency), 1 or 2 C4B genes in 63 normal siblings of patients with rheumatoid arthritis.

As with C4A, levels of C4B associated with 1 C4B gene (median 62.75mg/l; range 8.9 - 157.6mg/l; n=18) or 2 C4B genes (124.56mg/l; range 67.0 - 375.0mg/l; n=42) were statistically different (Mann-Whitney U=112, P<0.001) and there was a relationship between gene number and C4B concentration, however there was again marked overlap of phenotypic expression between the different genotypes (Fig 25).

The overlap between phenotypes was most marked when total C4 concentrations (C4d) were related to the number of C4A and C4B genes (Fig 26: C4d: 2 genes=128.75mg/l, range 60 - 150mg/l, n=7; 3 genes = 210.62mg/l, range 105.2 - 512.8mg/l, n=26; 4 genes=246.38mg/l, range 65.5 - 541.0mg/l, n=30). The differences between these groups did reach statistical significance (Kruskal-Wallis H=15.17, P<0.001)

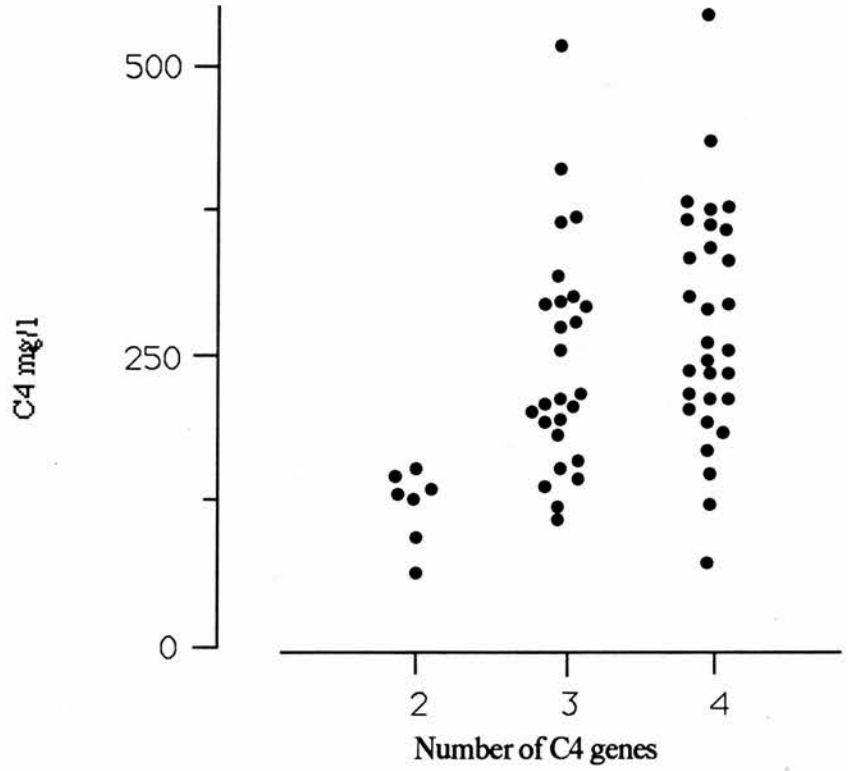

Figure 26. Concentration of total C4, measured as C4d, associated with 2, 3 or 4 C4 genes in 63 normal siblings of patients with rheumatoid arthritis.

3.3 Factors potentially contributing to variability of C4 gene expression

Gender

In mice the C4 homologue, Slp, is under androgenic control. I therefore looked for evidence that the expression of C4A or C4B in humans might be similarly controlled. Thirty two haplo-identical sibling-pairs of opposite sex were available for study. These individuals showed no clinical or laboratory evidence of rheumatoid disease, and all

sera were negative for CRP. The results of this analysis are shown in Figure 27, below. Statistical comparison by Wilcoxon Signed Rank Pairs test showed no differences in C4A or C4B levels between males and females (for C4A, z=0.334, P=0.38; for C4B, z=0.314, P=0.38).

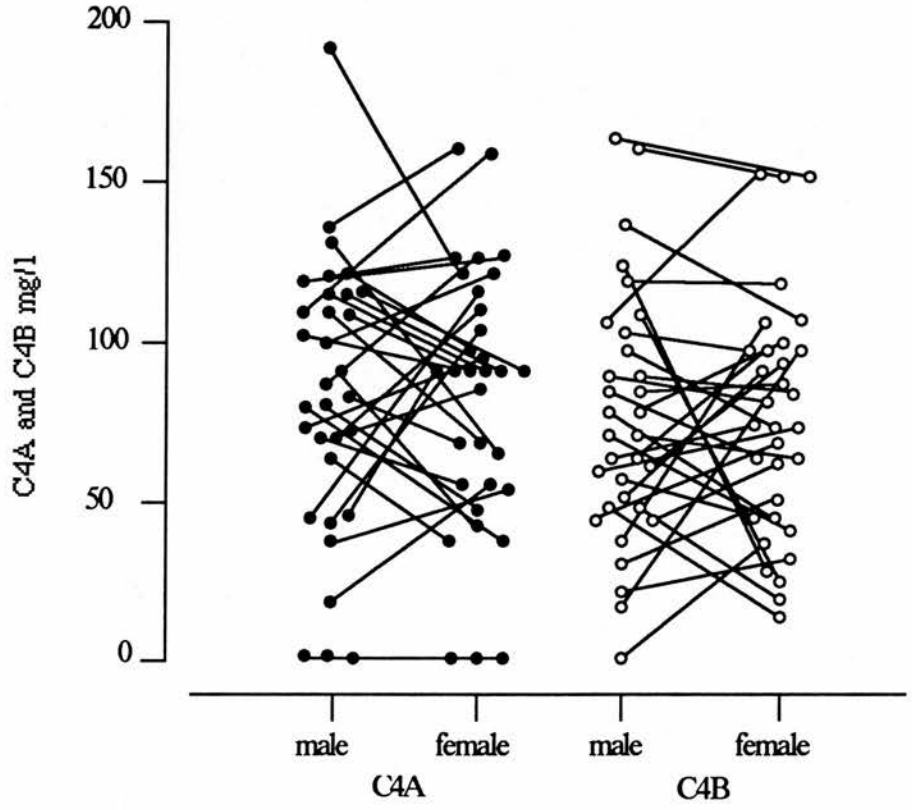

Figure 27. Concentration of C4A and C4B (mg/l) in 32 haplo-identical opposite sex sibling pairs. No statistical differences exist between the levels of C4A or C4B found in corresponding male-female sibling pairs.

The effect of null alleles on the expression of the other C4 isotype

There is some evidence for "feedback" control of synthesis of C4 *in vitro* [68]. I therefore tested the hypothesis that reduced C4 production due to a null allele of one isotype might be accompanied by increased synthesis of C4 of the other isotype. No such increase in synthesis was found. Subjects were identified with 2 C4B genes, accompanied by 0 (n = 7), 1 (n = 18) and 2 (n = 17) C4A genes. C4B expression was 103.5mg/l (range = 67.0 - 132.0mg/l) in subjects with homozygous deficiency of C4A compared with 123.4mg/l (range = 69.5 - 425.0mg/l) in those with 1 functional C4A gene and 141.9mg/l (range = 71.9 - 194.6mg/l) in those with 2 functional C4A genes (Kruskal-Wallis H=5.28, P=NS). In subjects with 1 C4B gene the concentration of C4B found in 4 individuals with 2 C4A genes was 74.5mg/l (range = 27.1 - 157.5mg/l) and in 14 individuals with 3 C4A genes was 60.38mg/l (range = 8.9 -

106.1mg/l). These are not significantly different (Mann-Whitney U=34, P=NS). Similarly, in subjects with 2 C4A genes, C4A expression was 120.2mg/l (range = 80.6 - 246.5mg/l) in 4 subjects with 1 C4B gene compared with 135.0mg/l (range = 44.8 - 200.9mg/l) in 17 individuals with 2 functional C4B genes (Mann-Whitney U=31, P=NS).

Age

Holme and colleagues [97] have reported that C4B levels rise with age by 12% per decade, while for C4A the increase is 3%. The effect of age was much less marked amongst the subjects studied here. The effect of age upon C4A levels in 31 subjects bearing 2 C4A genes is shown in figure 28A, while the relationship of C4B with age amongst 45 individuals with 2 C4B alleles is shown in figure 28B. Both show a rise with age but the effect is weak and is only significant in the case of C4B (C4B: r_S = 0.299, P = 0.046, C4A: r_S = 0.22, P = 0.23).

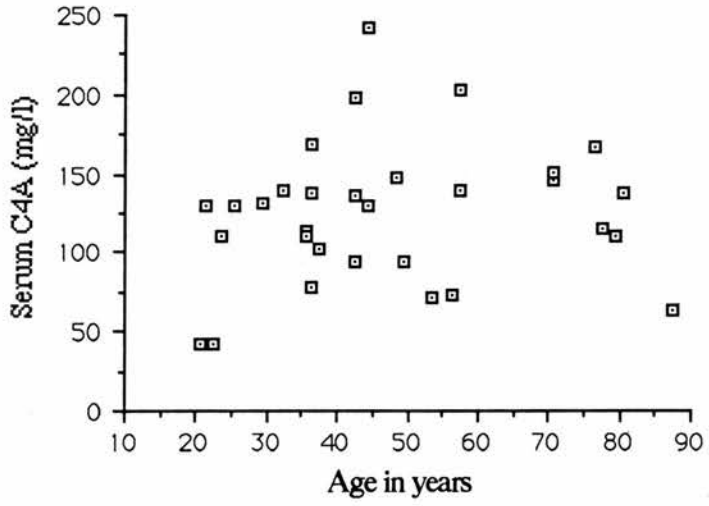

Figure 28A. The effect of age on C4A levels amongst 31 subjects with 2 C4A alleles.

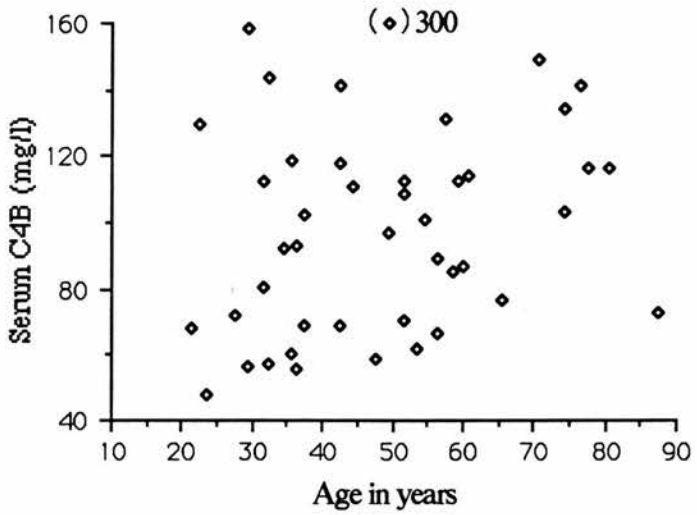

Figure 28B. The effect of age on C4B levels amongst 45 subjects with 2 C4B alleles.

Acute phase response

C4 is known to be an acute phase reactant [212]. It is not known whether C4A and C4B show differential responses to acute phase stimuli. I attempted to study this possibility by measuring C4 responses amongst 28 women (median age 59yrs) referred to the surgical unit of St Bartholomew's hospital for "lumpectomy" of breast lesions. All were subsequently shown to have benign disease. These subjects were chosen since they seemed unlikely to have a stimulus to complement consumption, reducing the confounding effect of combined alterations in C4 production and turnover and I am indebted to Mr M Woods and Dr C. Ingham-Clark for the collection of specimens and to Mr.C.Gilmore for allowing us to study his patients. Informed consent was obtained. Samples were taken on the day of admission, 1hr post-operatively, and at 24 and 48hrs post-operatively and were separated within 2 hrs of venesection and stored at -70°C prior to C4 estimation. For samples other than 1 hr post-operative, each patient was venesected at 11am after 1hrs bed rest. No clinical evidence of chest or post-operative wound infections was noted. CRP was kindly measured by the clinical chemistry department, St Bartholomew's hospital.

Twenty five patients showed a rise in CRP, the median levels at 0, 1, 24, and 48hrs being 5.0, 2.5, 47.0, 39.5 mg/l respectively. Serum C4d levels did not rise in the time-scale of these observations. Mean C4d was 190.7mg/l (+/-84.25 1SD) at time 0, 165.7mg/l (+/-76.5) at 1hr, 183.4mg/l (+/-89.4) at 24 hrs and 189.1mg/l (+/-92.6) at 48 hrs. There were no significant differences between these C4 levels and no correlation of C4d with CRP levels was found.

3.4 Measurement of C4 levels in individuals with haplotypes with anomalous antigenic expression

The apparent expression of C4A and C4B in 6 members of a family known to possess a C4B5 allele (Rodgers 1, Chido-1) (Dr C.Giles, personal communication) is shown in Table 7. In the family members with the MHC haplotype containing C4A4, B5,DR4 the C4 phenotypic levels are compatible with recognition of the C4B5 allele by the anti-Rodgers 1 antibody used in this study.

C4 alleles	C4A mg/l	C4B mg/l
A4, B5/A3, BQ0	155.0	0.0
A4, B5/A3, B1	201.60	65.87
A4, B5/A3, B1	167.25	67.75
A4, B5/A3, B1	129.87	55.87

Table 7. The apparent concentration of C4A and C4B associated with the complotype C4A4, C4B5 in 4 members of a family in whom the C4B5 allele was known to express the rare Rodgers 1 +ve, Chido 1 -ve serotype. Protein expressed by this C4B allele is recognised as C4A by the anti-Rodgers 1 monoclonal used to measure C4A in this study. For comparison, median C4 concentrations associated with 2 C4A and 2 C4B genes in this study were 134.8 and 124.5mg/l respectively.

Discussion

The immunogenetics of C4, and the relationship between C4 genotype and phenotype are complex. C4AQ0 are associated with SLE and a number of other diseases. It would be of use in epidemiological studies, and potentially in clinical practice, to be able to identify heterozygous deficiency of C4A by simple serum assays without the need for more complex genetic or complement allotyping procedures. I have therefore used the availability of isotype specific monoclonal antibodies to study the relationships between C4 genotype and phenotype, and factors affecting the expression of C4 alleles. Initial approaches to quantification of C4 isotypes depended on measurement of the ratio of C4A to C4B by densitometry, a method later refined by enzyme digestion of C4 which simplifies the banding pattern of C4 on gel electrophoresis [24]. Previous studies have shown a reduction of total C4 associated with the presence of null alleles [213]. Reference ranges for the identification of genetic deficiency have been suggested [214] and

densitometry of electrophoretic bands has been used to quantify C4 isotype levels and to estimate the presence of homoduplicated C4 loci [215]. Scanning densitometry may allow probable assignment of single null alleles [216], but will not detect the presence of null alleles at both loci. Accurate quantitation of C4A and C4B is now possible using monoclonal antibodies which are virtually specific for each isotype. However, results obtained by application of these reagents to sensitive ELISA assays [97,209] still showed overlap between phenotype and genotype, a finding which I have confirmed here. While an obvious dose effect of C4 gene number on mean C4 levels was seen when each isotype was considered separately (figures 24 and 25), the range of overlap between each group did not allow accurate assignment of genotype from the phenotypic data. Furthermore, when total C4 levels were measured, the distinction between groups was even less distinct (figure 26) and I have concluded that it is *not* possible to determine the presence of a single null allele from measurement of total C4.

The factors contributing to this phenotypic variability are not known, and it is not clear whether there is a quantitative polymorphism of C4 allele expression analogous to the erythrocyte complement receptor, CR1. Differential regulation of mouse C4 and Slp expression by tissue specific trans acting (non H-2-linked) genetic influences has been demonstrated [65,66], and variable expression of C4 in individuals known to have *identical* extended haplotypes may suggest similar mechanisms in humans [68]. The observation that the total C4 measured in individuals with 3 or with 4 C4 genes was similar suggested the possibility that there may have been partial compensation for the presence of a null allele by increased expression of the associated opposite isotype. However, I could find no evidence to support this hypothesis when I considered the effect of the presence of null alleles on levels of the opposite isotype.

Other physiological factors which may affect C4 expression include age, sex and the acute phase response. I could detect no effect of gender on C4 levels in haplo-identical brothers and sisters (figure 27), suggesting that human C4 levels are not influenced by sex hormone production. Holme and colleagues [97] have reported that C4B levels rise with age by 12% per decade, while for C4A the increase is 3%. The effect of age was much less marked in my data. Levels of C4A and C4B both tended to rise with age (figure 28A and 28B) but these correlations were weak and, in the case of C4A, did not reach statistical significance. C4 does respond as an acute phase protein and, in the mouse, C4 isotypes show differential responses to inflammatory stimuli [71]. Normal CRP levels in these sera make an acute phase response an unlikely cause of variation in this study. My attempt to study the acute phase responses of C4 isotypes directly was unsuccessful due to failure of C4 levels to rise within 48hrs of an adequate surgical stimulus. Little complement

consumption was anticipated amongst these patients undergoing relatively minor surgery with no evidence of sepsis. A statistically insignificant post-operative fall in C4 levels was noted and may suggest that complement consumption occured. Major trauma reduced C4 levels [217] and surgery involving cardiopulmonary bypass results in a transient fall in C4 levels with evidence of complement turnover [218], levels returning to normal after 48hrs. While I had not anticipated high levels of C-consumption in these patients undergoing more minor surgery, the similarity of the time scale of changes in C4 levels suggests that this might have occured. Alternatively, the time scale of these observations may have been inadequate since the progressive rise in C4 seen after acute myocardial infarction was maximal (140%) after 80 hrs [212].

Knowledge of the extended haplotype which is inherited in linkage with individual C4 molecules is of assistance in interpreting phenotypic and other data. RFLP analysis coupled with pulsed field gel electrophoresis [219] has shown that the C4BQ0 in the extended haplotype encoding B44, C4A3, C4BQO, DR4 is associated with the presence of a duplicated C4A gene in locus 2 (the C4B locus). The findings here (figure 24) confirm the observations [220] that this duplicated gene is expressed. This haplotype may be a further source of variability in studies in which extended haplotypes are not analysed. It is of particular interest that the B44, C4A3, C4BQO, DR4 haplotype is found at increased prevalence amongst patients with Felty's syndrome [210] and it has been suggested that the mechanism underlying the association in related to the role of complement in IC processing [210]. The case for an important physiological effect of the C4B deficiency encoded by this haplotype is weakened by the finding that this haplotype is associated with increased production of C4A, which would be expected to partially compensate for the C4B deficiency, especially with regard to immune complex opsonisation.

C4A and C4B are highly homologous proteins with less than 1% variation in total amino acid sequence between the two isotypes. Isotypic identity is conferred by substitutions amongst a group of 4 amino acids which lie in the C4d region of the α chain. As explained in chapter 1, the relationship between isotypic identity and serological activity is complex and a further 3 groups of amino acid substitutions involving 6 residues are also involved in determining antigenic reactivity (see figures 4, 5 and 6). Most C4A molecules express the Rodgers 1 antigenic determinants while most C4B molecules are recognised by antibodies to Chido 1. Monoclonal antibodies to these determinants allows the accurate measurement of immunological reactivity in serum which equates *almost* exactly with the presence of C4A and C4B. However, the correspondence between serological reactivity and isotypic identity is not absolute and rare alleles which express reversed antigenicity such as a Rodgers+1, Chido -ve C4B5 molecule have been recognised. In the observations reported

above (table 7), one family was known to posses this allele. The apparently anomolous levels of C4 found in 7 members of this family (table 7) illustrates the subtle difference between isotypic and serotypic identity and points to one source of spurious variability in studies of this type. Similar findings were made by Holme and colleagues in a family carrying a C4B5 allele and the opposite effect was noted with a C4A1 molecule [97].

To summarise these studies, I have found a high degree of variability in the expression of C4 genes in normal individuals, confirming previous observations and suggesting that there may be considerable difficulties in the accurate determination of C4 genotype from phenotypic data. Of the physiological variables considered, gender does not affect C4 expression, while age has only a small effect. I found no compensatory increase in the production of the opposite isotype in the presence of a null allele. The problems of reversed antigenic expression have been demonstrated and I have shown that the homoduplicated C4A gene in the Felty's syndrome associated haplotype A44, C4A3, C4BQO, DR4 is expressed.

Chapter 4

Experiments related to the radioligand binding enumeration of erythrocyte surface C4A, C4B, C4d and DAF

The conditions used here for radioligand binding experiments are the same as those used in previous studies of erythrocyte surface C3 deposition [175] and are known to produce 95% saturation of monoclonal anti-C3d (clone 3) and anti-CR1 (E11) in cell surface ligand binding sites. The experimental conditions and method employed are described above. However, the radioligand binding characteristics of Mab anti-Rg 1 (anti-C4A), Mab 1228 (anti-C4B), Mab T2. C5.12 (anti-C4d) and of anti-DAF have not previously been studied and a series of preparatory experiments were therefore performed.

4.1 Maximal binding of radiolabelled antibodies by excess erythrocytes

To determine the proportion of immunoreactive material in radiolabelled antibody preparations, the binding of anti-C4A, anti-C4B and anti-C4d with erythrocytes at a concentration 25 x the experimental radioligand binding conditions, was measured.

Method

Erythrocytes bearing high numbers of C4 molecules were prepared by incubation with pool serum at low ionic strength as described above. Controls were prepared by opsonising normal erythrocytes with low C4 surface deposition with serum, 10mM EDTA (for anti C4d binding), or with normal serum deficient in the C4 isotype to be measured (for C4A and C4B). Cells were washed 3 times in PBS, 1% BSA buffer, packed and used at the maximal concentration of 1.25 x 10^{10}/ml. Antibody and cells were incubated at 37°C for 30 minutes in duplicate and erythrocyte bound label separated from free by spinning duplicate 150µl aliquots through oil as described for radioligand binding assays.

Results

Maximal specific binding are shown below in table 8. The non-specific binding in each control sample has been subtracted.

Mab anti-Rg:1 (anti-C4A)	**32.37%**
Mab 1228 (anti-C4B)	**20.56%**
Mab T2.C5.12 (anti-C4d)	**68.26%**

Table 8. maximal specific binding of radiolabelled anti C4 antibodies to excess EC4A, EC4B and EC4.

Discussion

These values are a slight underestimate of maximal binding achievable, since a small number of both C4 isotypes are present on the control erythrocytes (approximately 1% of the measurable C4 surface molecules recognised on the test cells). The background count in radioligand binding studies is primarily due to non-specific trapping of label amongst the erythrocytes during centrifugation and rises with the density of cells in the reaction. Measurement of background at high cell concentrations in this way may thus lead to an overestimation of the non-specific binding present under experimental conditions. However, a second experiment with anti-C4B at 4% of the above cell concentrations (equivalent to the relative concentrations in radioligand binding assays) showed similar results with 20.79% specific binding despite 20-fold reduction in background These results demonstrate the total percentage of functional antibody binding activity in the radiolabelled preparations used in these studies though the exact proportion in successive aliquots of antibody would be likely to vary depending on radiation damage during the radiolabelling reactions and, potentially, with the homogeneity achieved by separate antibody purification procedures.

4.2 Saturation of ligand sites in radioligand binding studies

It is important to use saturating quantities of antibody in order to achieve accurate quantification of cell surface ligand sites. The concentration of antibody in the reaction mixture in these ligand binding experiments was 5μg/ml, as previously described [175]. Figure 29, overleaf, shows the effect of alterations in the concentration of anti-C4A, anti-C4B and anti-C4d under standard reaction conditions with normal control erythrocytes from an individual HB known to have serum concentrations of 25.0mg/l of C4A and 70.31mg/l of C4B and low numbers of erythrocyte C4A and C4B (35 and 39 molecules/cell respectively).

Method

Serial dilutions of each antibody in PBS/BSA buffer were prepared starting from 50μg/ml. 75μl aliquots were incubated with 375μl of erythrocyte suspension (at $4x10^8$/ml) giving maximal reaction concentration of antibody of 8.3mg/ml. After incubation, E-bound antibody was separated from free and bound counts corrected for non-specific background as described above.

Results

The results of this experiment expressed as ng of antibody specifically bound in each reaction are shown below in Figure 29.

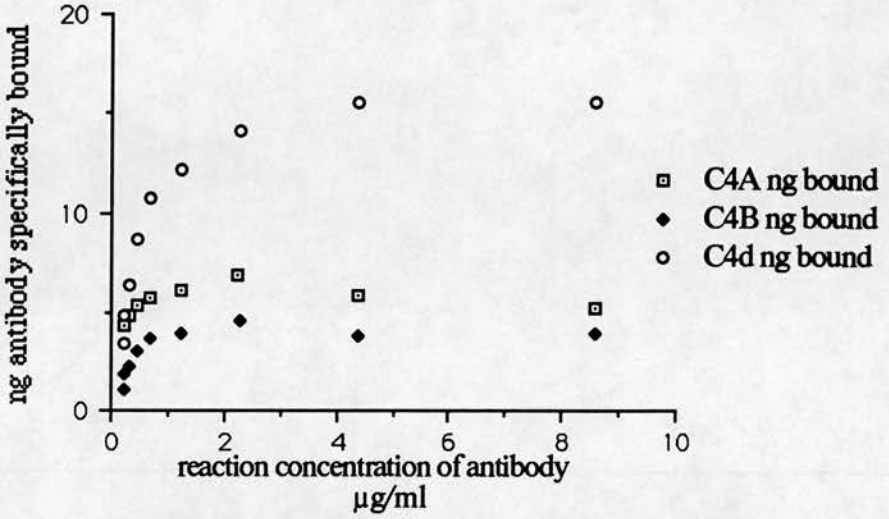

Figure 29. The relationship between the reaction concentration of anti-C4A, Anti-C4B and anti-C4d and the quantity of antibody (in ng) specifically bound to erythrocyte surface ligands. Saturated binding is found at a reaction concentration of 0.5µg/ml for anti-C4A and Anti-C4B and approximately 1µg/ml for anti-C4d. The reaction concentration used in radioligand binding assays was 5µg/ml.

Discussion

It can be seen that specific erythrocyte antibody binding is saturated in the range from 10µg/ml to approximately 0.5µg/ml (1µg/ml for anti-C4d). At the reaction concentration used (5µg/ml) the quantification of ligands is unlikely to be significantly affected by the differences in % total binding activity of radiolabelled antibody preparations noted above, or by small variations in the quantity of antibody offered in each reaction due to experimental error.

4.3 The specificity of C4 isotype recognition by Mab anti-Rg:1 (anti-C4A) and Mab 1228 (anti-C4B) in radioligand binding assays

The specificity of recognition of C4A an C4B by Mab Anti-Rg:1 and Mab 1228 respectively, is of critical importance in the accuracy of the results reported here. The binding specificity of these antibodies have been independently verified. In addition, the aliquots of antibody donated for these experiments were tested by the semi-quantitative differential agglutination method (kindly performed by Dr C. Giles). However, it was important to exclude the possibility that very low levels of cross recognition of C4 isotypes could affect quantification in sensitive radioligand binding assays.

Method

Normal blood group O erythrocytes bearing approximately 100 C4A and 400 C4B molecules/cell were coated with C4A or C4B by incubation at low ionic strength with normal serum deficient in the isotype to be measured. 75µl aliquots of 8 serial dilutions of antibody giving an initial reaction concentration of 10µg/ml were incubated with 375µl aliquots of washed erythrocytes at a reaction concentration of $7.5x10^8$/ml, then bound antibody separated from free and non-specific background subtracted as before. Both cell substrates were probed with both antibodies. Results are shown below in figs 30 and 31.

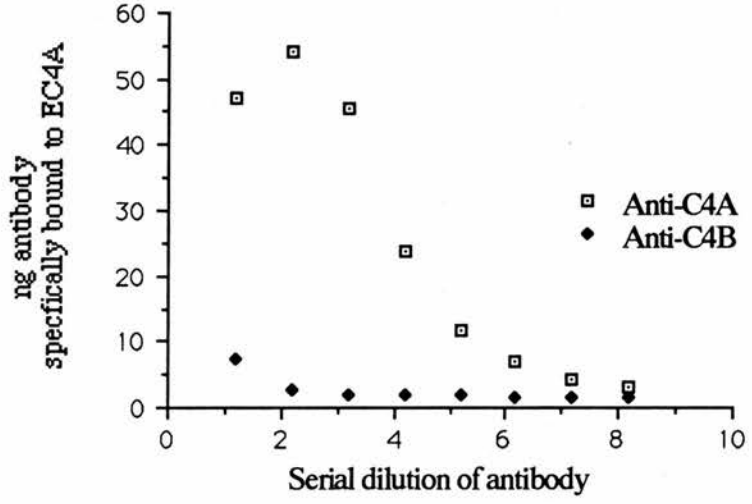

Figure 30. Comparison of anti-C4A and anti-C4B binding to EC4A. Reaction concentration of antibody in the first serial dilution is 10µg/ml.

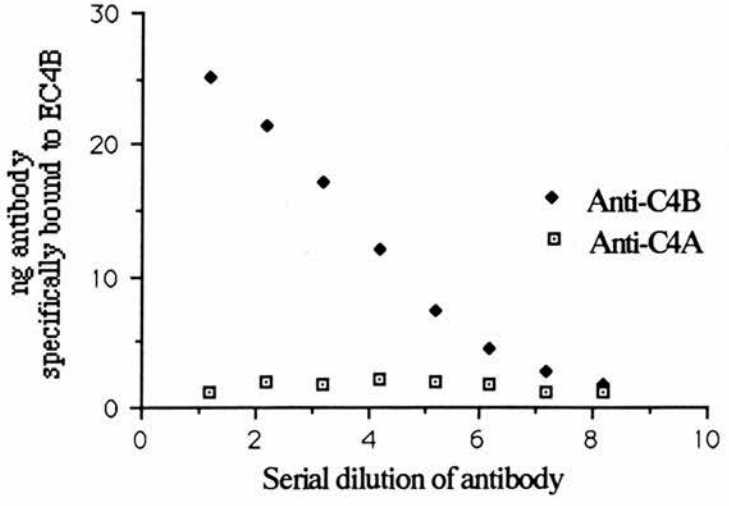

Figure 31. Comparison of anti-C4A and Anti-C4B binding to EC4B. Reaction concentration of antibody in the first serial dilution is 10µg/ml.

Discussion

It can be seen from figures 30 and 31 that there is little evidence of cross recognition of opposite isotypes by the C4A and C4B monoclonal antibodies used. A low level of specific binding of the opposite isotype was seen and was appropriate to the numbers of molecules of that isotype present on normal erythrocytes used to prepare EC4A and EC4B. There may, however, be a measurable recognition of C4A by mab 1228 at the highest concentration of antibody used (figure 30). Given the conditions of this experiment which employed cells with very high C4A numbers and higher concentrations of all reagents than in standard assays (equivalent to 10 - 16,000 molecules/cell) , these low levels of cross-reactivity are unlikely to be of significance under routine conditions.

4.4 Scatchard analysis of anti-C4A, anti-C4B, and anti-C4d binding to erythrocytes

Since the use of these monoclonal antibodies has not previously been described in radioligand binding assays, analysis of antibody affinity by Scatchard binding plots [221] was undertaken. In general, I felt that the most accurate measurement of affinity was likely to be obtained by assay of anti-C4A and anti-C4B binding in the absence of the opposite isotype on the erythrocyte surface. It was also important to the validity of C4 quantification that the anti-C4d monoclonal used here recognised C4A and C4B with the same affinity. I therefore prepared EC4A and EC4B cell substrates, in addition to EC4 and normal cells in the Scatchard experiments performed. Lastly, there was the potential problem of variation in binding affinity due to radiolabelling damage of any individual aliquot of antibody unduly influencing the results obtained in any single experiment. Therefore, Scatchard binding experiments were repeated under slightly different experimental conditions and using aliquots of antibody from at least 2 separate radiolabelled batches (3 for anti-C4B and anti-C4d) to obtain mean estimates of antibody affinity.

Method

Cell substrate and control cells varied according to the relevant conditions for each experiment and are summarised along with results in table 9:A, and B (p115, 116) and Scatchard plots are shown in figure 32: A1-3, B1-4 and d1-4 (pp117-119). C4A bearing erythrocytes (EC4A) used in experiments B4, and d4 were prepared with cells from a C4A deficient normal donor opsonised with C4B deficient serum. The control was anti-C4A binding, and C4B binding was adjusted for this value after allowing for differences in specific activity of the antibodies. Experiments A1, B1 and d1 were performed with E from a normal donor bearing 102 C4A and 329 C4B molecules cell and had no formal internal control. In experiments A2 and d2 these erythrocytes were coated with C4A from donor deficient serum by incubation at low ionic strength, while in experiments B2 and d3

these erythrocytes were coated with C4B and control values in the presence of excess (20μg/ml) "blocking" unlabelled anti-C4B was subtracted.

In each instance a 75μl aliquot of serial dilution of radiolabelled antibody was incubated with erythrocytes and controls at the concentrations indicated, prior to separation of bound from free as before. Results were expressed as bound/free cpm and as moles of antibody specifically bound/L of reaction volume. Binding affinity constant was calculated as the negative slope of regression. Experimental results are summarised in table 10, below.

Results.

The mean affinities calculated in this way are shown below in table 10

Mab anti-Rg:1	**(anti-C4A)**	**8.17×10^8 M^{-1}**
Mab 1228	**(anti-C4B)**	**4.07×10^8 M^{-1}**
Mab T2. C5.12	**(anti-C4d)**	**1.058×10^9 M^{-1}**

Table 10. Mean binding affinity constants of monoclonal anti-C4 antibodies.

Discussion

A number of these experiments employ erythrocytes substrates bearing both isotypes. The demonstration (see above) that there is negligible cross recognition of C4A and C4B by the monoclonal antibodies used here implies that an accurate estimate of antibody affinity may be obtained despite the presence of small numbers of molecules of the opposite isotype. The erythrocytes used in experiment B1 bear more C4A than C4B and a high relative concentration of antibody (17μg/ml) was employed. In addition this experiment returned the highest value for C4B antibody affinity (6.07×10^8 M^{-1}). Some cross recognition of C4A may therefore have been measured. Omission of this result produces a small reduction of the calculated mean affinity of Mab 1228 to 3.41×10^8 M^{-1}. A variety of controls were used in these experiments. In the absence of significant cross-reactivity between Mab anti-Rg1 and Mab 1228, the main effect of background subtraction is to shift the position of the line in the Scatchard plot, but not to alter the slope. Validation of the C4 measurements made in these (and other) experiments depends on the correlation of total C4 measured as the sum of individual isotypes with total C4 measured by Mab T2. C5.12, anti-C4d. Experiments d2, d3 and d4 were performed with EC4A (d2) and EC4B (d3, d4) substrates and show no significant difference in the affinity of anti-C4d for each isotype. The affinity of anti-C4d with EC4A was 1.197×10^9 M^{-1} and the mean affinity for EC4B in experiments d3 and d4 was 1.213×10^9 M^{-1}.

Table 9A: Summary of Scatchard binding analyses of Mab anti-C4A and anti-C4B binding affinity

Antibody and aliquot number	Maximum reaction concentration of antibody (µg/ml)	Reaction concentration of erythrocytes (x 10^8/ml)	Cell substrate and controls	Measured affinity x 10^8 M/L	Figure number
Anti-C4A					
A1*	6.0	10.0	Normal E with 102 C4B and 329 C4A/cell	**9.11**	A1
A2**	2.5	3.33	EC4A: normal E with 102 C4B/cell) opsonised with B null sera (C4A 521Z/cell)	**5.06**	A2
A1§	1.0	1.0	SLE E with high C4A(879/cell) and C4B (648/cell)	**10.37**	A3
Anti-C4B					
B1*	17.0	5.0	Normal E with 102 C4B/cell	**6.07**	B1
B2***	6.0	7.5	EC4B: normal E opsonised with C4A null serum Control blocked with 20µg/ml unlabelled anti-C4B	**3.00**	B2
B1§	1.0	1.0	SLE E Control: unlabelled blocking anti-C4B at 20µg/ml	**5.73**	B3
B4¶	8.0	1.07	EC4B: A null cells opsonised with autologous A null serum Control: EC4B probed with anti-C4A	**1.49**	B4

Results obtained in the same experiment are marked *, **, ***, § and ¶

Table 9A.. For legend see text.

Table 9B: Summary of Scatchard binding analyses of Mab anti-C4d binding affinity

Antibody and aliquot number	Maximum reaction concentration of antibody (µg/ml)	Reaction concentration of erythrocytes (x 10^8/ml)	Cell substrate and controls	Measured affinity x 10^8 M/L	Figure number
Anti-C4d					
d1**	2.5	3.33	Normal cells with 263 C4d/cell	**6.07**	d1
d1***	6.0	7.5	EC4A: Normal E opsonised with C4B null sera	**11.97**	d2
d3***	6.0	7.5	EC4B: Normal E opsonised with C4A null serum. Control blocked with 20µg/ml unlabelled anti-C4B.	**13.40**	d3
d5¶	8.0	1.07	EC4B: A null cells opsonised with autologous A null serum Control: EC4B probed with anti-C4A	**10.86**	d4

Results obtained in the same experiment are marked *, **, ***, § and ¶

Table 9B.. For legend see text.

Figure 32 A1 - A3:
Scatchard binding analyses of Mab anti-C4A binding affinity

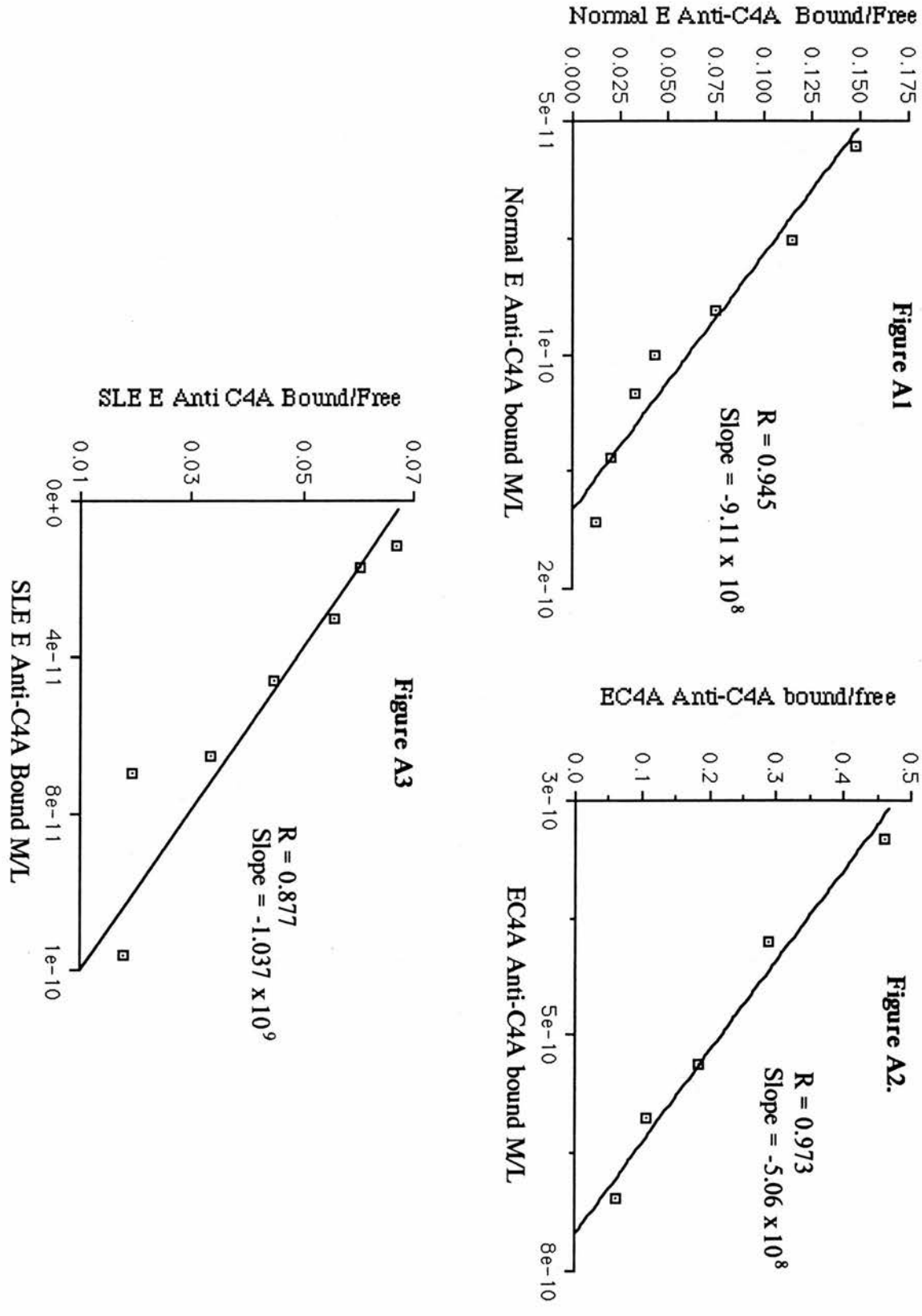

Figure 32 A1 - A3. for legend see text.

Figure 32 B1 - B3:
Scatchard binding analyses of Mab anti-C4B binding affinity

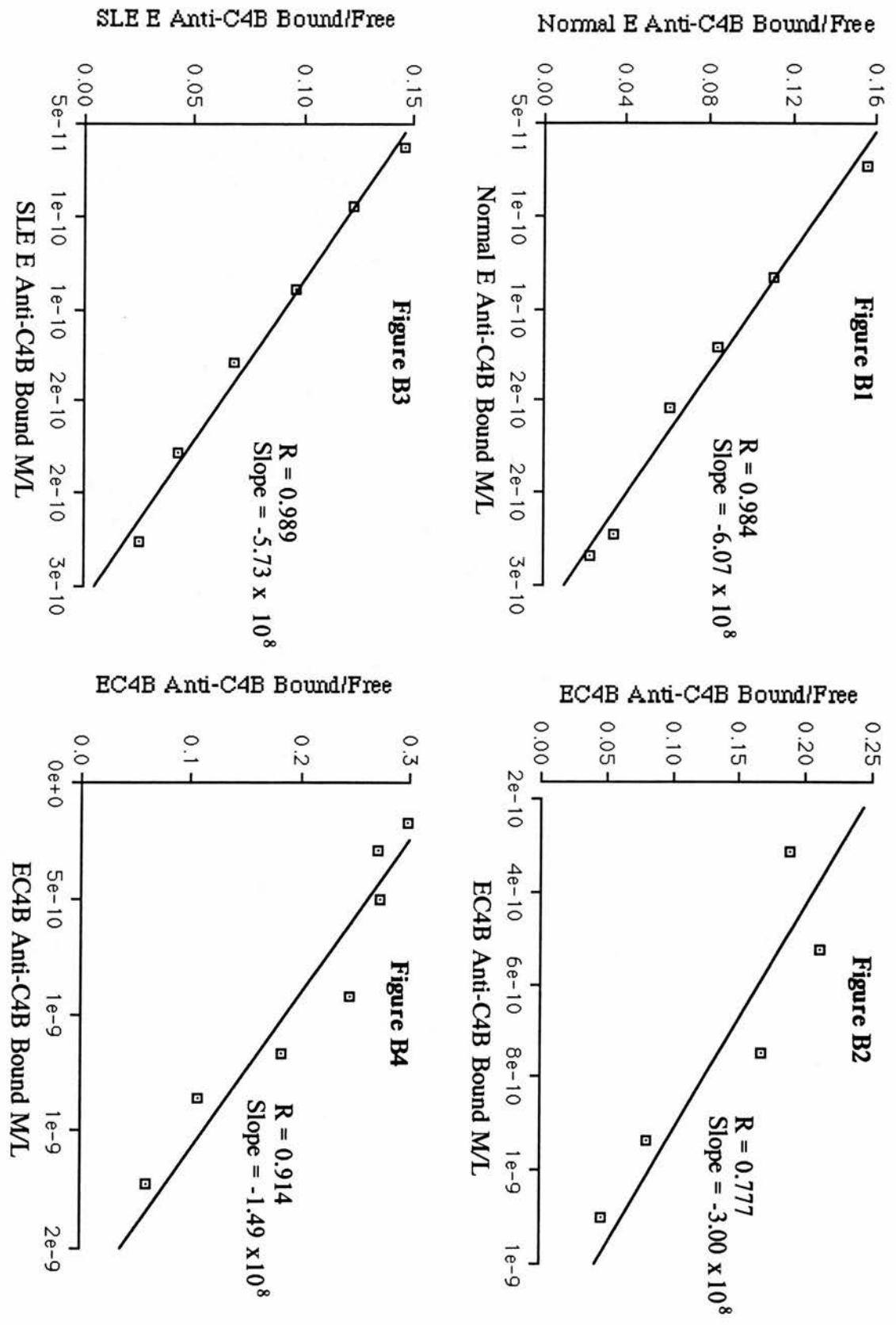

Figure 32 B1 - B3. for legend see text.

Figure 32 d1 - d3:
Scatchard binding analyses of Mab anti-C4d binding affinity

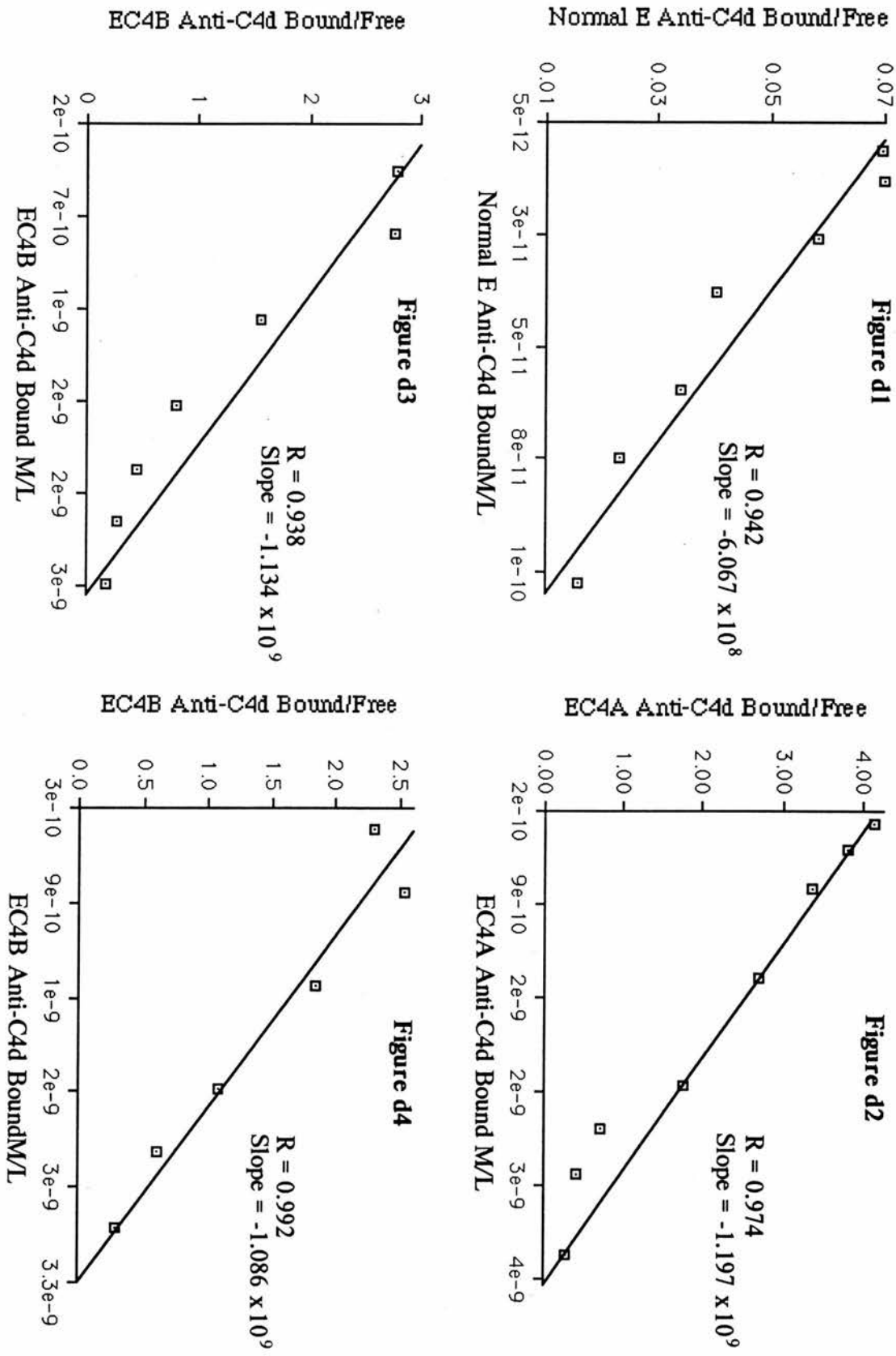

Figure 32 d1 - d3. for legend see text.

4.5 Correction of C4B enumeration

During the course of the experiments above (and others), it became apparent that Mab 1228, anti-C4B appeared to systematically under-read the number of C4B molecules present on test erythrocytes. For example, EC4A used in experiments A2 and d2 showed equivalent numbers of C4 molecules when probed for C4A and for C4d (quantified as 5212 molecules of C4A/cell by Mab anti-Rg:1 and 4439 molecules of C4d/cell by Mab T2. C5.12). By comparison, EC4B (bearing approximately 300 C4A/cell) used in experiments B2 and d3 were measured as 1301 C4B and 3181 C4d by MAbs 1228 and T2. C5.12 respectively. Experiments were therefore performed to quantify this effect.

Method

A series of erythrocytes with increasing numbers of C4 molecules/cell were prepared by incubating with Blood group O normal erythrocytes with C4A and C4B from serial dilutions of donor deficient serum in low ionic strength buffer. A 375µl aliquot of cell suspension was then incubated with 75µl of antibody of known specific activity followed by separation of bound from free in the usual way. The binding of anti-C4A with EC4A was measured concurrently by two separately radiolabelled aliquots of anti-C4A [anti-C4A (9) and anti-C4A (10), respectively], and with one aliquot anti-C4d [anti-C4d (9)], while EC4B were measured with two preparations of anti-C4B [anti-C4B (8) and anti-C4B (9)], respectively] and with the same preparation of anti-C4d. Counts specifically bound were corrected for background binding as described in general methods. Specific binding is expressed as µg of antibody bound/reaction. Cell numbers in each reaction were checked prior to incubation and did not show significant variation.

Results

Regression analysis of antibody binding in these experiments are shown in figure 33A and 33B (overleaf).

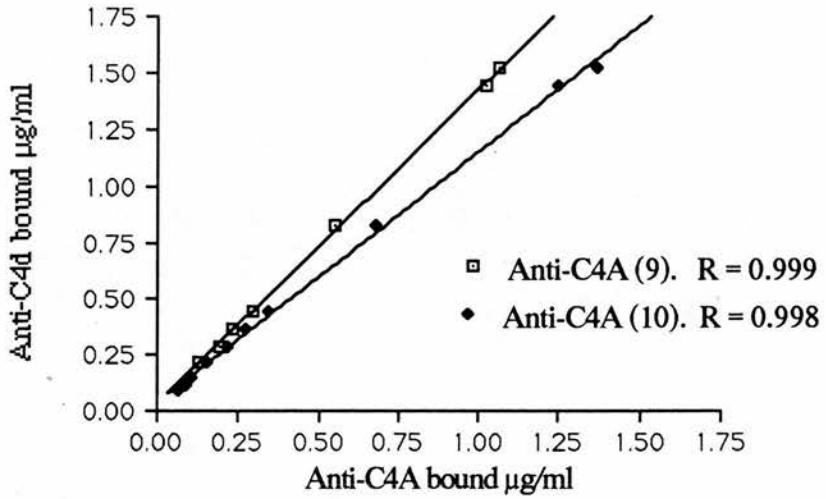

Figure 33 A. Regression analysis of specific binding of 2 radiolabelled aliquots of anti-C4A [anti-C4A (9) and anti-C4A (10)] with one radiolabelled aliquot of anti-C4d [anti-C4d (9)] with EC4A expressed as µg bound/ml reaction mixture.

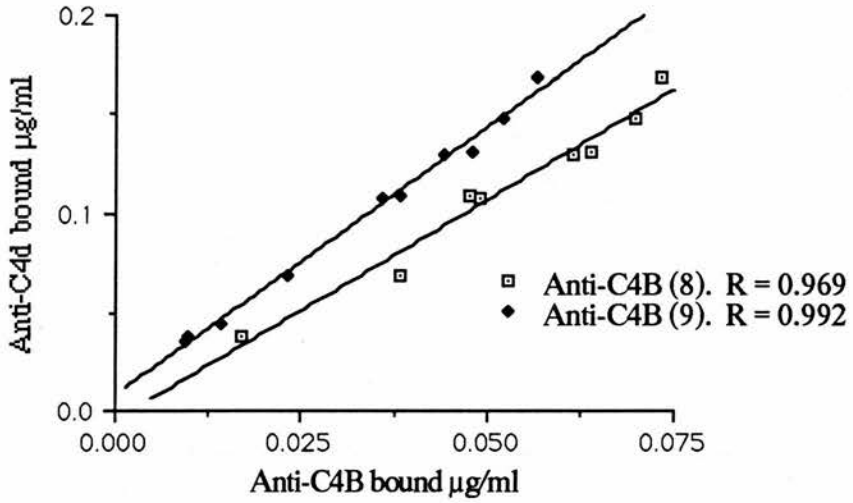

Figure 33 B. Regression analysis of specific binding of 2 radiolabelled aliquots of anti-C4B [anti-C4B (8) and anti-C4B (9)] with one radiolabelled aliquot of anti-C4d [anti-C4d (9)] with EC4B. Expressed as µg bound/ml reaction mixture.

The regression slopes are shown below in table 11.

Antibody	Slope of regression
Anti-C4A (9)	1.39
Anti-C4A (10)	1.11
Anti-C4B (8)	2.24
Anti-C4B (9)	2.71

Table 11. Slopes of linear regression analysis of 2 aliquots of anti-C4A [anti-C4A(9) and A(10)] and 2 aliquots of anti-C4B (anti-C4B(8) and B(9)] with 1 aliquot of anti-C4d [anti-C4d(9)].

Discussion

It will be seen that while the regression of C4A with C4d is near unity the regression of both aliquots of anti-C4B demonstrates a systematic reduction in the quantification of cell surface C4B molecules by this antibody (discussed below). These and a number of other experiments (data not shown) confirmed that Mab 1228 recognised only a proportion of the C4B molecules present on the cell surface. A correction factor of 2.5 (the mean regression of anti-C4B in these experiments was 2.48) was therefore applied to the numbers of C4B molecules measured on experimental samples. This figure may be slightly high since the EC4B used in the experiments described here bore a small number of C4A molecules (100/cell) which would be recognised by anti-C4d but not Mab 1228 and therefore tend to increase the regression slope. However the number of C4B molecules on each cell was such that this effect is small, and has not been considered further.

4.6 Validation of cell surface C4B correction factor

Evidence that this correction is valid may be obtained by considering alterations in the linear regression analysis of the sum of C4A and C4B with C4d molecules/cell measured on erythrocytes before and after the application of the correction for reduced C4b recognition. Erythrocytes were obtained from 68 individuals in order to study differences between erythrocyte surface C4A and C4B deposition *in vivo*. Figure 34, overleaf shows the results of regression analysis before and after application of this correction factor to these data.

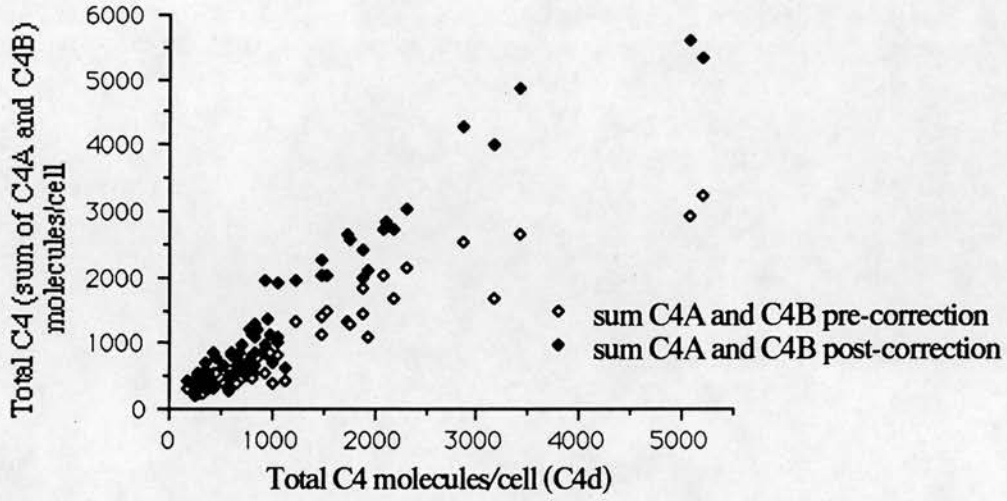

Figure 34. Linear regression analysis of total numbers of C4 molecules/cell measured as the sum of individual C4A and C4B measurements and C4 total measured as C4d before (pre-correction) and after (post-correction) the application of the calculated correction factor of 2.5 to the estimates of C4B. Correlation coefficients are 0.931 and 0.967 pre and post-correction respectively. Regression slopes are 0.69 and 1.29 pre and post-correction respectively.

Discussion

Figure 34 demonstrates improvement in the accuracy of the regression of sum C4A and C4B with C4d after correction of C4B enumeration in this way and all experimental results are reported after application of this correction factor. This analysis also confirms the internal consistency of the measurement of erythrocyte surface C4 sites in that total C4 measured as the sum of the two isotypes correlates closely with total C4 measured as C4d across the whole range of C4 numbers measured.

4.7 Studies with Anti-DAF: Scatchard analysis of binding affinity

Method

Initial experiments demonstrated that a starting concentration of 6.667 x 10^7E/ml (reaction concentration 3.333 x 10^7/ml; 1 x 10^7 cells/aliquot counted) was optimal for DAF enumeration. This is equivalent to 20% of the standard cell concentration employed for the other radioligands. Scatchard binding was performed in the conventional manner by incubating 75µl aliquots of serial dilutions of ^{125}I anti-DAF, initial concentration 15µg/ml, with 375µl aliquots of test normal erythrocytes bearing 1420 measured DAF sites/cell at 37°C for 30 minutes prior of separation of bound from free by centrifugation.

Results

Results were expressed as bound/free cpm and specific bound antibody in moles/L of reaction mixture and the binding affinity constant derived from the negative regression slope of the Scatchard plot as illustrated below in figure 35.

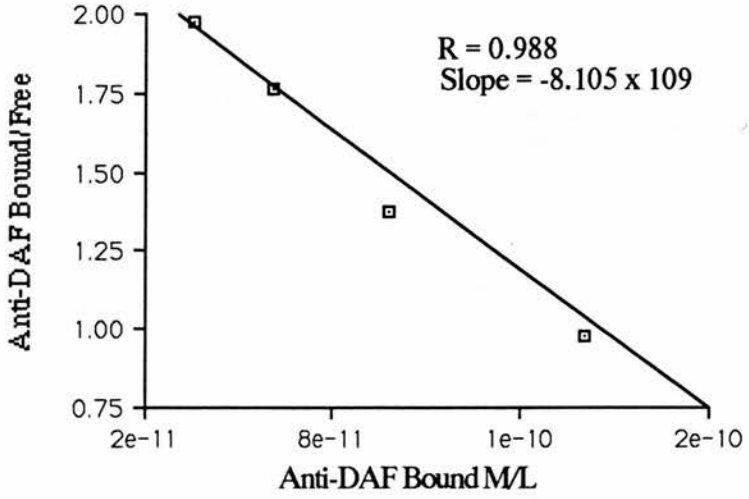

Figure 35. Scatchard binding plot of monoclonal anti-DAF.

The binding affinity constant of monoclonal anti-DAF was

$$8.105 \times 10^{9}\ M^{-1}$$

4.8 Saturation of cell surface ligand sites by mab anti-DAF

An experiment was performed to ensure that cell surface ligand binding was saturated at the concentration of antibody offered in the reaction mixture.

Method

Serial dilutions of anti-DAF were incubated with test erythrocytes from a normal control subject under standard conditions. The numbers of erythrocytes in each reaction tube were counted prior to incubation and did not vary significantly.

Results

The results are shown overleaf in figure 36 expressed as the apparent number of DAF molecules/cell

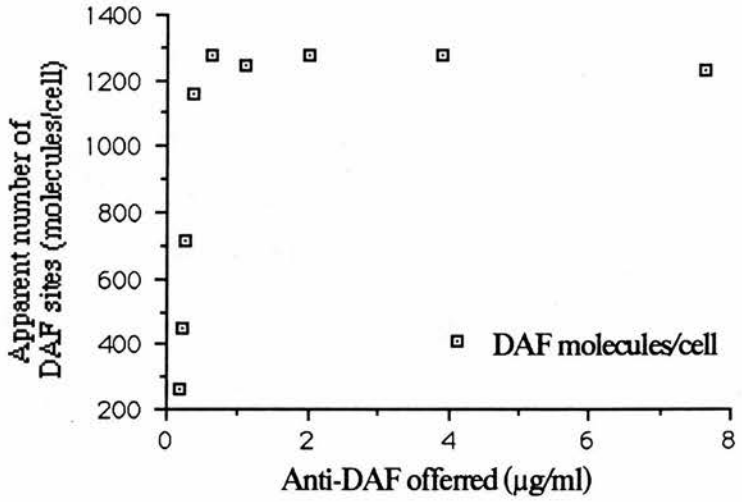

Figure 36. Binding of anti-DAF from serial dilution with normal erythrocytes after incubation under standard conditions expressed as apparent numbers of molecules/cell.

Discussion

It can be seen from figure 36 that the enumeration of DAF molecules was constant in the range of anti-DAF antibody concentrations 8.0 - 0.5µg/ml antibody offered. Experiments were therefore conducted with anti-DAF at 2µg/ml starting concentration (0.333µg/ml reaction concentration).

General Discussion

These experiments have explored the application of monoclonal antibodies (anti-C4A, anti-C4B, anti-C4d and anti-DAF) to radioligand binding enumeration of these cell surface antigens in preparation for their use in studies of the pathophysiology of C4 isotypes in SLE. Optimal assay conditions and the binding affinity constants of these monoclonals have been defined. One major problem was encountered, that Mab 1228 appears to systematically undercount the number of erythrocyte surface C4B molecules. The principal evidence for this was derived from comparison of the concurrent enumeration of erythrocyte-bound C4A (on C4B deficient EC4A) by anti-C4A and anti-C4d, with similar measurements of EC4B made concurrently by anti-C4B and anti-C4d. Measurement of C4A and C4d were equivalent while Mab 1228 showed a systematic reduction of C4B enumeration across a wide range of antigen concentrations (figure 33A and B, table 11).

The explanation for this is not clear. Damage to the monoclonal during radiolabelling is possible, though if so, the effect was consistent throughout a number of repeated

radiolabelling procedures. The affinity constant of Mab 1228 ($4.07x10^8M^{-1}$) was lower than the C4A and C4d monoclonals ($8.17x10^8$ and $1.058x10^9M^{-1}$, respectively), though this was not a very marked difference. Nor was any difference in binding affinity of anti-C4d towards C4A or C4B found in Scatchard binding studies (table 9A and B), excluding the possibility that the problem was due primarily to variation in the affinities of the *other* anti-C4 monoclonal antibodies employed. Iodination of the antibody may have affected antigen recognition, or there may exist more than one antigenic population of C4B on erythrocytes, though this seems unlikely. Mab 1228 was purified from ascites and may therefore have been contaminated by light chain or irrelevant immunoglobulin. The low maximal binding acheived with this antibody (20.56%, table 8) may suggest that this was so, though moab anti-C4d was also purified from ascites by the same procedures and showed much higher maximal erythrocyte binding (68.26%, table 8). The use of antibody at non-saturating concentrations could contribute to undercounting, though initial experiments seem to have excluded this possibility (figure 29). Whatever the cause, application of the correction factor derived from a number of experiments including those shown in figures 33: A and B correct the undercounting and improve the correlation of total C4 measured as C4A and C4B with total C4 measured as C4d (figure 34) and I have therefore felt it justified to correct the experimental erythrocyte C4B measurements by application of this factor.

Chapter 5

Erythrocyte C4, C3 and DAF deposition in patients with SLE and the primary antiphospholipid (1° APL) syndrome *in vivo* and Immune Complex C4 deposition *in vitro*

Efficient disposal of immune complexes *in vivo* requires classical pathway activation. Patients with SLE are at risk of functional C4 depletion due to genetic deficiency of C4 alleles, especially C4A. Functional differences between C4A and C4B may therefore be of importance in the pathophysiology of SLE. C4A binds preferentially to amino groups and thus to protein antigens, while C4B binds preferentially to carbohydrate moieties and thus to erythrocytes. Detailed understanding of these functional differences has arisen primarily from studies of a small number of purified C4 isotypes, individually or mixed, in serum free conditions *in vitro* [37-43]. I therefore studied the immune complex binding activity of C4A and C4B in two contrasting models: binding to SLE erythrocytes *in vivo* (erythrocyte surface antigens are primarily carbohydrate) and binding to test immune complexes of protein antigen (heat aggregated IgG) from complex mixtures of whole SLE sera *in vitro*.

5.1 Studies with SLE erythrocytes *in vivo*

Erythrocyte complement deposition is a common abnormality in patents with SLE. Mechanisms contributing to this *in vivo* are likely to include complement fixation secondary to direct autoantibody binding and bystander deposition during erythrocyte-mediated CIC processing. Similar mechanisms are likely to affect patients with the 1° APL syndrome. The greater affinity of the C4B intrachain thiolester for hydroxyl moieties is the principal determinant of increased *sheep* E surface deposition and haemolytic activity *in vitro*. However, C4A and C4B binding with *human* erythrocytes *in vivo* may be modulated by the presence of regulatory proteins such as DAF.

The demonstration that an excess of C4B over C4A is found on SLE erythrocytes would suggest that these physiological differences between C4 isotypes are of importance *in vivo* as well as *in vitro*. I therefore studied Erythrocyte surface C4A, C4B, (C4d) C3d and DAF numbers and compared these with the concurrent serum C4A and C4B (and C4d) concentrations in subjects with SLE, the primary antiphospholipid syndrome and normal controls.

Patients

Pathological samples were obtained from 49 subjects including 45 (1 male) conforming to ARA criteria for definite SLE and 4 (1 male) with findings suggestive of the primary anticardiolipin syndrome (for simplicity these are referred to as SLE sera in figure legends). 19 normal control specimens were obtained from clinically healthy laboratory staff (8 males and 11 females). Erythrocyte surface DAF numbers were measured in 27 of these patients and in 18 of the healthy controls.

Samples

In each case, two 10 ml samples of blood were taken. Serum and erythrocytes were prepared as described in general methods (chapter 2). In all cases, serum samples were refrigerated at -70°C within 4hrs of venesection. Erythrocytes were separated, washed and stored at 4°C in buffer within 4 hrs of venesection and were retained at 4°C for up to 72hrs prior to analysis.

Methods

Radioligand binding assays for the enumeration of erythrocyte surface C4A, C4B, C4d and C3d and RIA of serum C4A, C4B (and C4d) levels were performed as described in general methods (chapter 2). Non parametric methods were used for all statistical comparisons and results are quoted as median and range where appropriate.

Results

5.11 Serum and erythrocyte C4A and C4B

Results of the measurement of C4A and C4B in these sera are shown overleaf in figure 37 Amongst control subjects, median serum C4A concentration was 91.2mg/l (range: 0.0-286.25mg/l), while C4B concentration was 100.0 mg/l (0.0-251.25mg/l). In subjects with SLE median serum C4A concentration was 48.8mg/l (0.0-185.0mg/l), while C4B was 66.2mg/l (6.2-251.2mg/l).

Erythrocyte C4A and C4B levels in these subjects are shown in figure 38: A and B. Amongst control subjects, the median number of C4A molecules/erythrocyte was 62.0mols/cell (range: 5.0-236.0mols/cell), while the median number of C4B molecules/erythrocyte was 198.4mols/cell (0.0-568.9mols/cell). Amongst subjects with SLE, the median number of C4A molecules/erythrocyte was 297.0mols/cell (1.0-1796.0mols/cell), while median C4B mols/cell was 749.1mols/cell (54.9-4382.2mols/cell).

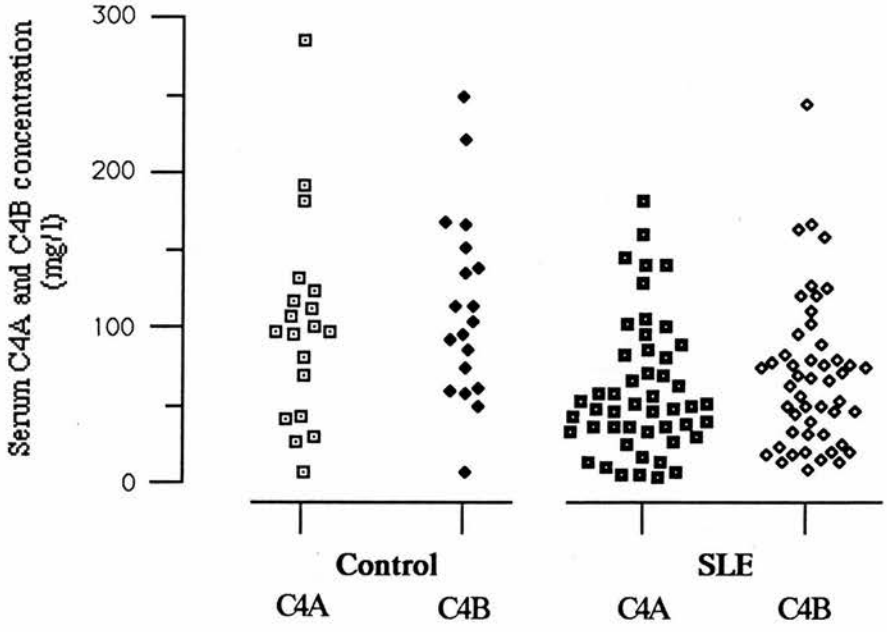

Figure 37. Serum C4A, C4B concentrations (mg/l) amongst 49 patients with SLE and the primary APL syndrome and 19 normal controls. For legend , see above

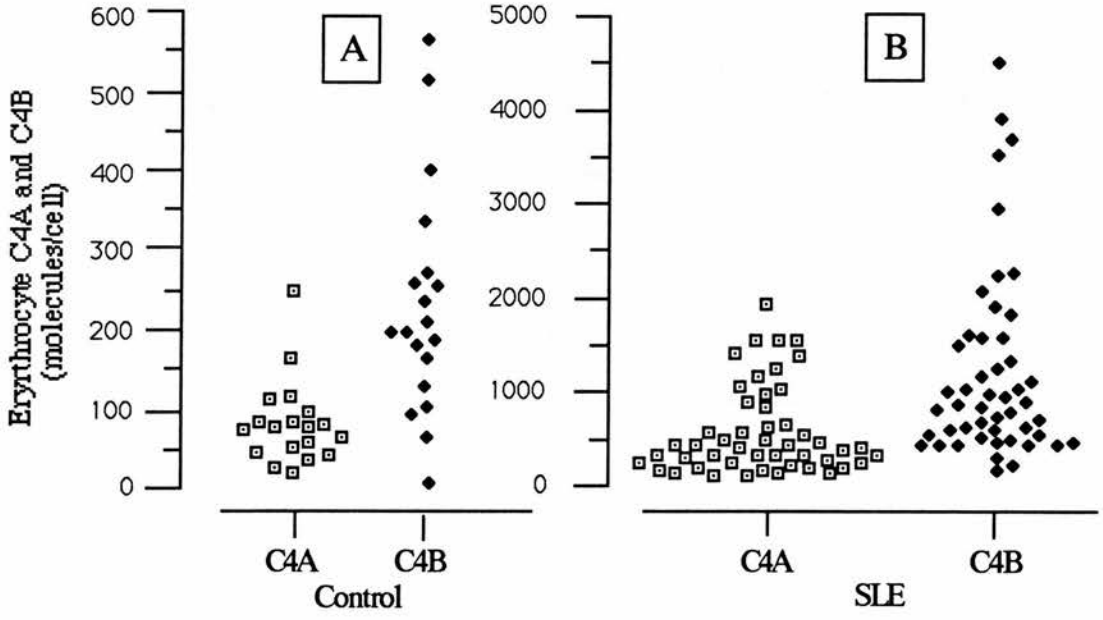

Figure 38: A and B.. Erythrocyte cell surface C4A and C4B (molecules/cell) amongst 19 normal control subjects (figure 38: A) and 49 subjects with SLE and the primary APL syndrome (figure 38: B). For legend, see above

Statistical differences amongst these data were sought by the Mann-Whitney U test. Table 12, below, shows the results.

Comparison	Control (n=19)		SLE (n=49)	
	U	P	U	P
Serum C4A/C4B	**201**	<0.274	**1348**	**<0.0002**
Erythrocyte C4A/C4B	**318**	<0.108	**1790**	**<0.0001**

Table 12. Mann-Whitney U comparison of serum and erythrocyte levels of C4A and C4B amongst 19 control subjects and 49 subjects with SLE and the 1° APL syndrome.

Discussion

A Statistically significant excess of C4B was found on SLE erythrocytes in these studies (C4B 749.1mols/cell: range 54.9-4382.2 ,C4A 297mols/cell: range 1.0-1796.0).However, a significant reduction of serum C4A concentration was also present in SLE sera (C4A 46.8mg/l: range 0.0-185.0, C4B 63.6mg/l: range 6.2-241.2) and could have been responsible for the erythrocyte findings. I therefore sought correlations between serum and erythrocyte C4A and C4B levels to investigate this possibility.

5.12 Correlation of serum and erythrocyte C4A and C4B levels

Results of the correlation of serum and erythrocyte C4A levels amongst control subjects are are shown overleaf in figure 39 and the same analysis for C4B in figure 40. Correlation of serum and erythrocyte C4A and C4B levels amongst SLE subjects are shown in figures 41 and 42 (p132). Results of Spearman correlation analysis are shown below in table 13.

Correlation	Control		SLE	
	r_s	P	r_s	P
serum/erythrocyte **C4A**	0.595	< 0.007	0.276	<0.055
serum/erythrocyte **C4B**	0.451	<0.053	-0.039	<0.079

Table 13. Correlation of serum and erythrocyte C4A and C4B amongst 19 normal control and 49 subjects with SLE and the 1° APL syndrome.

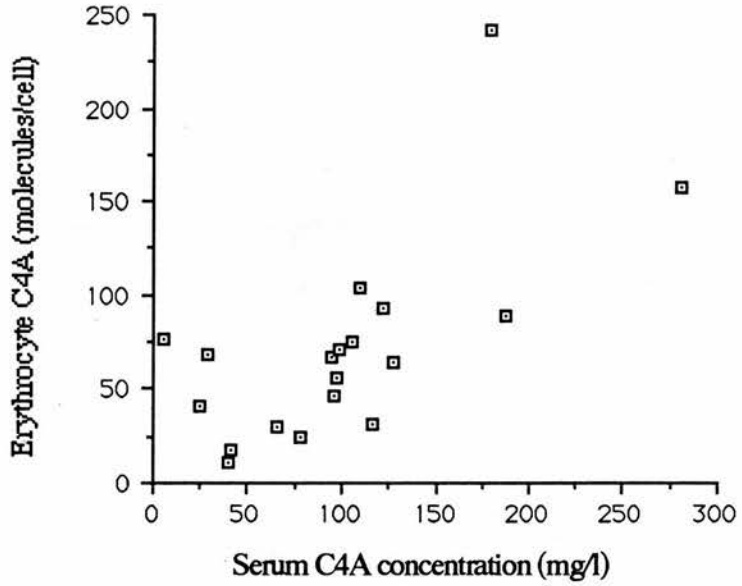

Figure 39. Correlation of serum C4A concentration with erythrocyte C4A levels amongst 19 normal control subjects. Spearman correlation was 0.595, P = <0.007.

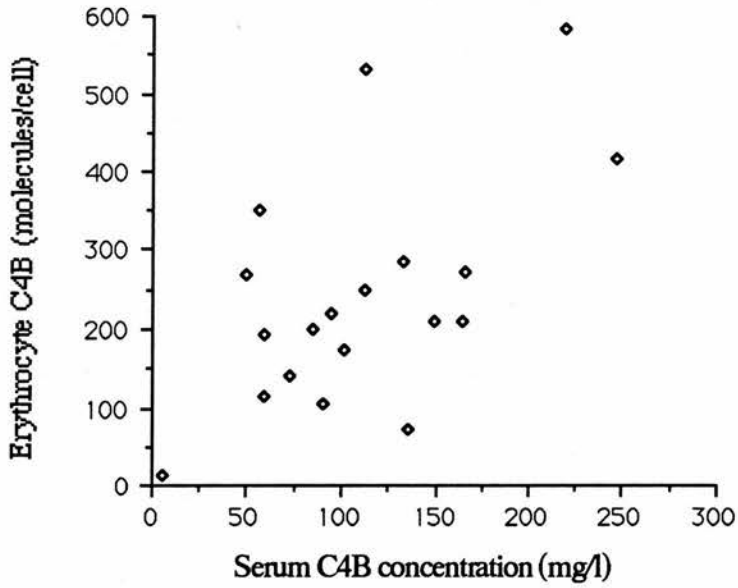

Figure 40. Correlation of serum C4B concentration with erythrocyte C4B levels amongst 19 normal control subjects. Spearman correlation was 0.451, P = <0.053.

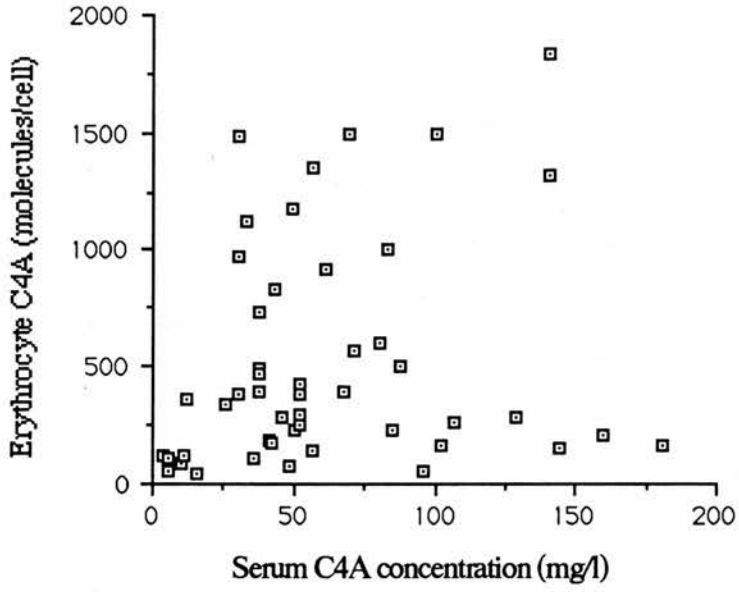

Figure 41. Correlation of serum C4A concentration with erythrocyte C4A levels amongst 49 subjects with SLE and the 1° APL syndrome. Spearman correlation was 0.276, P = <0.055.

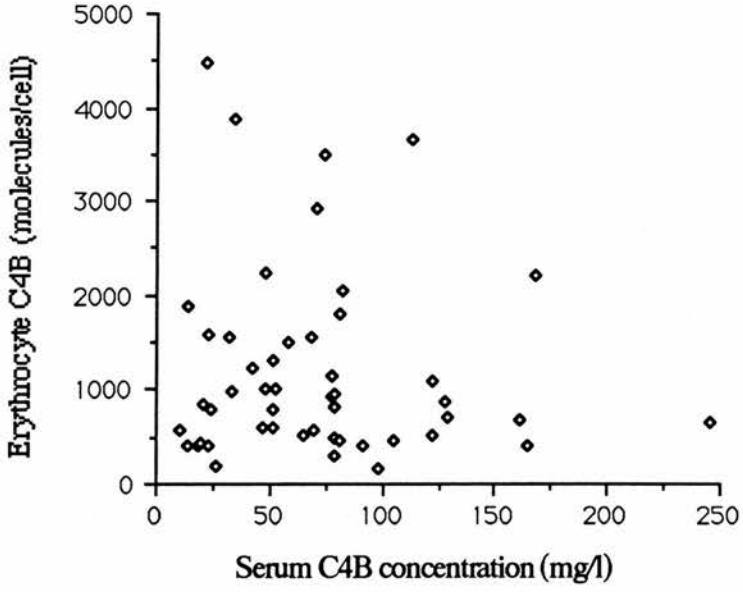

Figure 42. Correlation of serum C4B concentration with erythrocyte C4B levels amongst 49 subjects with SLE and the 1° APL syndrome. Spearman correlation was -0.039, P = <0.790.

Discussion

Amongst normal subjects there existed a strong correlation of serum C4A and C4B concentration with erythrocyte surface deposition (figures 39 and 40), though the correlation for C4B did not reach statistical significance amongst the small number of control subjects in this study. However, no correlations were found between serum and erythrocyte C4A and C4B levels in SLE subjects (figures 41 and 42). For C4B, a weak negative correlation (-0.039) was found (figure 42). These data suggest that reduction of serum C4A concentration is not responsible for the observed excess of C4B on SLE erythrocytes.

5.13 Studies of cell surface DAF

The cell surface regulatory molecule DAF could be important in determining differences in cell surface C4 deposition. I therefore measured erythrocyte surface DAF numbers were in 27 subjects with SLE and the primary APL syndrome and 18 normal control subjects by radioligand binding assay as described above.

Results

Numbers (median and range) of DAF molecules/erythrocyte amongst 27 subjects with SLE and the primary APL syndrome were **1369.0** (range 751.0-2109.0) and amongst 18 normal control subjects were **1324.5** (range 625.0-1780.0). These results are shown below in figure 43. These are not significantly different (Mann-Whitney U=229, P=NS)

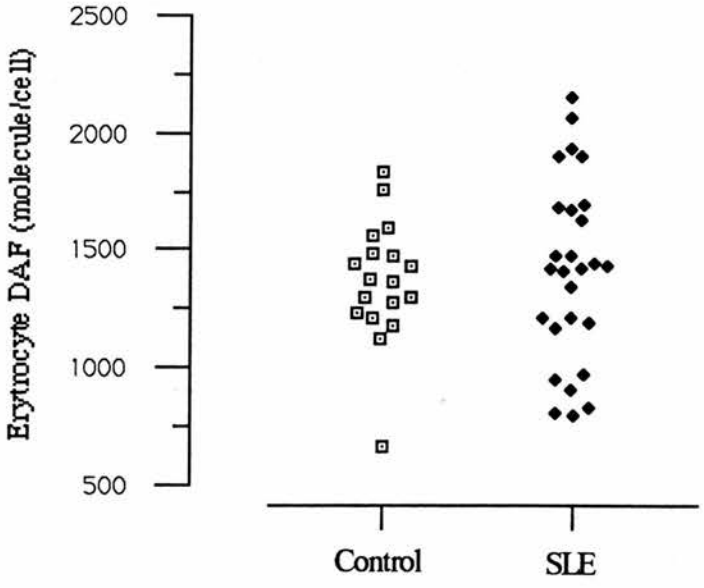

Figure 43. Numbers of erythrocyte cell surface DAF molecules amongst 27 patients with SLE (and 1° APL syndrome) and 18 normal control subjects.

5.14 Correlation of C4A and C4B with erythrocyte C3d deposition

Differences between C4A and C4B in their efficiency as mediators of classical pathway activation were sought by correlation of C4 isotype deposition with C3d on erythrocytes from patients with SLE and the primary antiphospholipid syndrome. Cell surface C4B shows a strong correlation with C3d deposition (figure 44: $r_S = 0.710$, P = <0.001) and approached a linear relationship (R = 0.849). However, the correlation of C4A deposition with C3d, though statistically significant, is less marked, (figure 45: $r_S = 0.380$, P = 0.007) and is only poorly modelled by linear regression (R = 0.376).

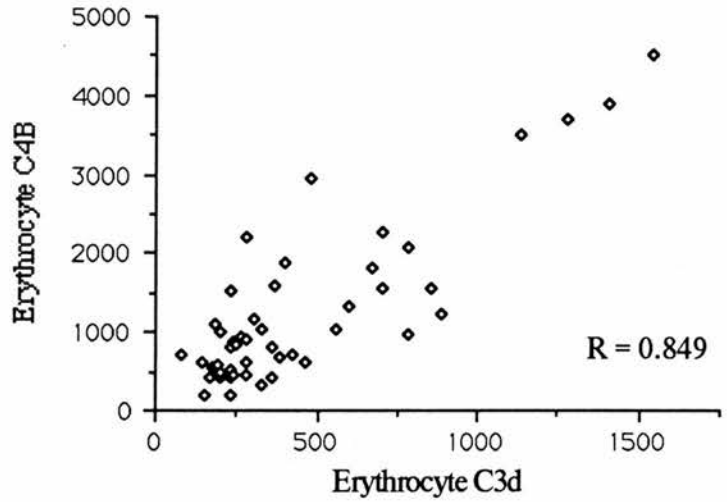

Figure 44. Correlation of erythrocyte surface C4B and C3d deposition amongst 49 patients with SLE and the primary antiphospholipid syndrome ($r_S = 0.710$, P = <0.001).

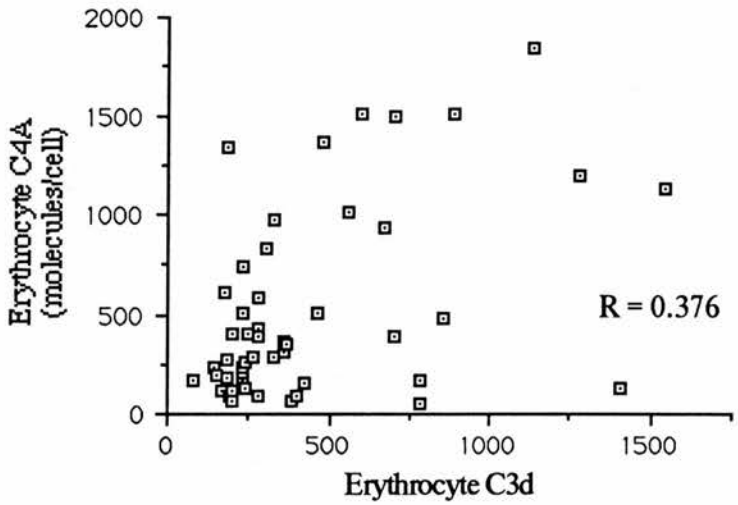

Figure 45. Correlation of erythrocyte surface C4A and C3d deposition amongst 49 patients with SLE and the primary antiphospholipid syndrome ($r_S = 0.380$, P = 0.007).

5.2 Quantification of C4 deposition on HAGG immune complexes *in vitro*

Erythrocyte surface antigens are predominantly carbohydrate in composition. Having studied differences between C4A and C4B in binding to erythrocytes *in vivo,* I then tested the binding of C4 isotypes to test immune complexes of protein antigen. The theoretical prediction from consideration of the differential ligand binding affinity of C4A and C4B is that C4A would show increased binding to ICs of protein antigens, the mirror image of the situation with erythrocytes. Studies utilising individual purified C4 isotypes *in vitro* [42,43] have tended to confirm this prediction, suggesting a ratio of 1.5-2.0/1 for the excess C4A binding. However, it is unclear whether the same relationships would be found when complement activation occurs in the presence of both isotypes and regulatory proteins *in vivo.* The fluid phase opsonisation of test immune complexes (heat aggregated IgG) by C4 isotypes in complex mixtures in whole serum was used as a model of the physiological situation and the relationship between serum C4 isotype levels and their deposition on test immune complexes was investigated using the assays for fluid phase (RIA) and IC-bound (ELISA) C4 described in chapter 2.

Patients and materials

Forty four subjects with SLE were studied. Sera were subjected to centrifugation at 10,000G twice after separation to remove pre-formed ICs and subaliquoted within 4hrs for storage at -70°C. Control opsonisation of HAGG was measured in the presence of 10mM EDTA, to exclude those samples with high levels of pre-formed circulating ICs. Five patients showed high levels of control binding (>10% pool serum equivalent) and were omitted from further analysis. Control binding was subtracted from all measurements prior to further analysis. A further 5 samples with C4AQ0 were excluded from analysis since this experiment was aimed at testing IC opsonisation in the presence of both isotypes, and also because the presence of zero values in both serum and IC assays produced spuriously high statistical correlations amongst C4A measurements. Spearman rank order correlation analysis was employed to test the strength of association between serum and IC C4 levels amongst the 34 samples remaining. For the purposes of comparison, both quantities were expressed as relative values, serum C4 as % pool serum and HAGG C4 levels as % pool serum equivalent (the same pool serum was used in both assays).

Results

5.21 Validation of HAGG C4 assays

Figure 46, below, shows the regression analysis of total C4 deposited on HAGG measured as the average of the independent measurements of C4A and C4B with total C4 measured as C4d.

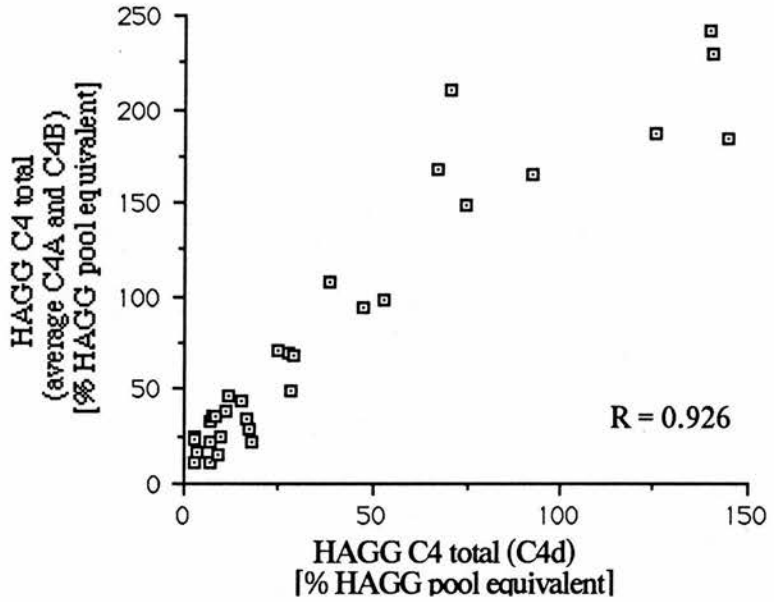

Figure 46. Validation of immune complex C4 quantification by linear regression of total C4 measured as the average of independently measured C4A and C4B with total C4 measured as C4d amongst 34 subjects with SLE.

5.22 Quantification of serum and IC C4 levels and their correlation

Table 14 (overleaf) shows the levels of C4 isotypes in serum and deposited on HAGG from these samples, the ratio of HAGG to serum levels derived from the median values and also the results of Spearman rank order correlation analysis of relationships between serum and IC C4 levels are also shown. Figure 47 A and B overleaf illustrate the lack of correlation between serum and IC C4A and C4B levels.

	C4A	C4B
	Median (Range)	Median (Range)
Serum C4 (% pool serum)	**40.83** (3.6-200.3)	**54.34** (8.7-316.0)
HAGG C4 (% pool serum equivalent)	**35.48** (0-328.8)	**38.22** (5.3-236.47)
Correlation Serum/HAGG levels		
r_S	**0.442**	**0.004**
P	**0.01**	**0.342**

Table 14. Quantities of C4A and C4B in serum and deposited on test immune complexes (HAGG) *in vitro* amongst 34 subjects with SLE, along with the results of Spearman rank order correlation analysis of serum and HAGG C4A and C4B levels.

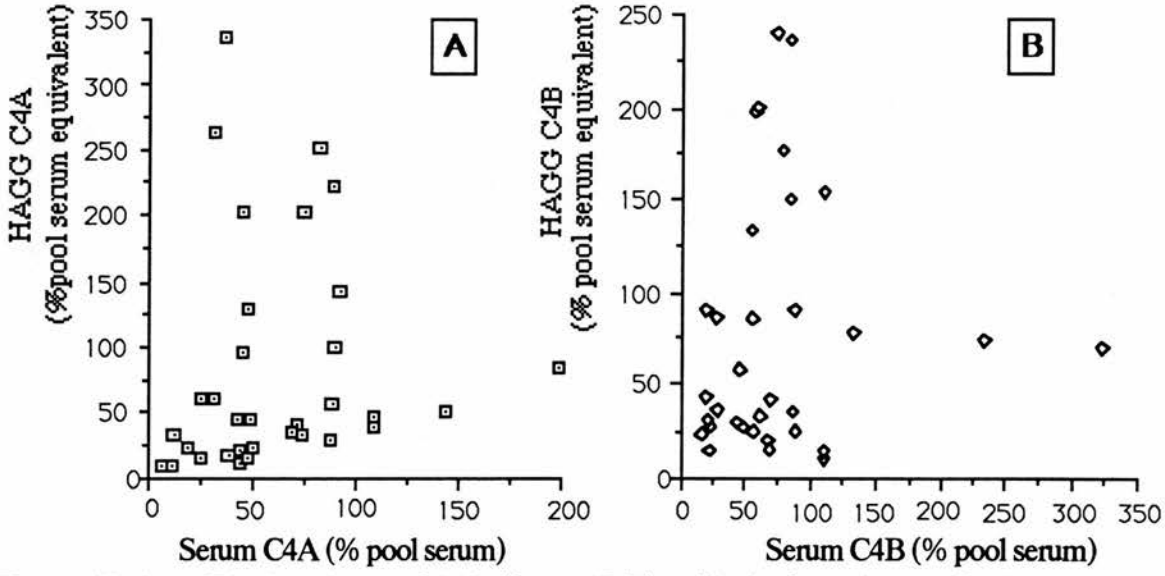

Figure 47: A and B. Correlation of C4A (figure 47 A) and lack of correlation of C4B (figure 47 B) deposition on HAGG with concomitant serum levels amongst 34 patients with SLE (see table 14, above for correlation coefficients).

Discussion

These experiments explored differences between C4A and C4B in erythrocyte and protein immune complex binding assays. Such differences may be determined primarily by differences in C4 isotype thiolester binding specificity and are likely to reflect important aspects of C4 physiology *in vivo.*

The first finding of these studies has been confirmation *in vivo* of the increased binding of C4B to erythrocytes (figure 38 A and B). Serum concentrations of C4 are equal amongst control subjects, but serum C4A concentrations are significantly lower than C4B amongst subjects with SLE (figure 37, table 12). That reduction of serum C4A does not account for the excess of C4B seen on SLE erythrocytes is demonstrated by the correlations of serum and cell surface C4A and C4B shown in figures 39-42, summarised in table 13. While a strong correlation of serum and erythrocyte C4A and C4B is seen in normal subjects (figures 39 and 40, for C4A: r_S=0.595, P=<.007, for C4B: r_S=0.451, P=<.053), this correlation is lost amongst subjects with SLE (figures 41 and 42, for C4A: r_S=0.276, P=<.055, for C4B: r_S=-0.039, P=<.079) and C4B shows a weak negative correlation. These data imply that reduced serum concentration of C4A did not determine the increased C4B deposition found on SLE erythrocytes The negative correlation of serum C4B with erythrocyte levels may imply that cell surface deposition is an important factor in reduction of serum C4B concentration in SLE, though this negative correlation did not reach statistical significance (table 13). Studies of cell surface DAF numbers show no difference between SLE and normal control subjects (figure 43), implying that variation in this cell surface complement regulatory protein was not a factor in determining pathological erythrocyte C4 deposition in SLE *in vivo.* In fact, weak negative correlation of DAF levels on cell surface deposition of C4 were found, though these were not statistically significant (data not shown). These findings confirm and expand those of previous *in-vitro* studies and are consonant with the known preferential binding of the C4B intrachain thiolester to the carbohydrate moieties abundant on the erythrocyte surface.

In addition to increased numbers of C4B molecules on the erythrocyte surface, my data may be taken to suggest that C4B forms a more efficient erythrocyte bound C3 convertase. While cell surface levels of both isotypes correlate with C3d deposition, the correlation of C4B (r_S = 0.710, P = <0.001) is stronger than C4A (figure 45, r_S = 0.380, P = 0.007), and approached a linear relationship (R = 0.849, figure 44).

The known increased affinity of C4A for amino-group ligands is paralleled by the greater efficiency of C4A than C4B in the opsonisation of protein ICs for CR1 binding *in vitro*

[42], and this may be the functional link which underlies the association of C4AQ0 with SLE. The hoped-for corroboration of this difference in the experiments described here, using mixtures of varying proportions of C4A and C4B in whole SLE serum as a model of the *in vivo* situation, was lacking. I found a weak, though just statistically significant, correlation of serum C4A levels with IC C4A deposition (figure 47A, table 14: for C4A r_S = 0.442, P = 0.01), while serum C4B levels did not appear to be directly correlated to IC deposition (figure 47B, table 14: for C4B r_S = 0.04, P = 0.342). The possibility that correlations would be apparent at lower serum C4 concentrations was examined in subgroups of the data, but no consistent effects were found. It may be argued that the approach taken here is unlikely to demonstrate subtle effects. Schifferli [40] found that mixing C4A and C4B from single donor deficient sera masked the excess activity of C4A in IIP *in vitro* and that the addition of even 1% C4 to serum free systems restored full IIp activity. In the light of these facts, my data may be interpreted as *tending* to support a more important role for C4A. However, viewing the data displayed graphically in figures 47A and B underlines the weak overall correlation between serum C4 levels and IC isotype deposition and an alternative conclusion is that factors other than serum C4 levels are of importance in modulating the opsonisation of ICs in *whole* serum.

While these experiments give no information as to the nature of such additional factors, a number of possibilities exist. C4 is a labile protein and even in carefully collected samples their may be artefactual loss of C4 *functional* activity reducing the sensitivity of the simple comparative approach employed here. My techniques also specifically removed (in the preparation of serum from plasma) and controlled for, the presence of naturally occurring ICs, which may have had some effect on the levels of functional C4 remaining for experimental activation. Isenman and Young [38] found no differences between C4A and C4B in their activation by purified C1q, however, recent studies [222] have found differences in the levels of C4 activation products associated with the presence of C4AQ0, which appeared to be independent of the associated C4 levels. Thus differences in the serum levels of other complement proteins may be a biological factor contributing to variability in these experiments. Carefully timed, dynamic studies may have demonstrated subtle differences in the opsonic efficiency of C4A and C4B. Preliminary experiments of this kind measuring C4A and C4B binding to HAGG at intervals demonstrated that opsonisation was complete after 1 minute, but no consistent differences between C4A and C4B could be found (data not shown).

Results

Chapter 6

Reduced numbers of CR1 on erythrocytes from patients with SLE and the antiphospholipid syndrome are associated with increased levels of anticardiolipin antibodies

CR1 displays a numerical polymorphism on erythrocytes which is controlled by both inherited and acquired factors. Amongst normal humans, the number of CR1 molecules on erythrocytes varies between means of approximately 100 and 1500 molecules per cell [102]. The level of numerical expression is stable amongst normal subjects and is, in part, regulated by a locus encoded in close vicinity to the CR1 structural gene on Chromosome 1 [125]. It has been shown that CR1 numbers are reduced on the erythrocytes of patients with several diseases including SLE [168-170,172,175] autoimmune haemolytic anaemias [175], paroxysmal nocturnal haemoglobinuria [223], AIDS [224], and lepromatous leprosy [225]. There has been dispute whether the mechanism for this reduction amongst SLE patients is inherited or acquired. Evidence for the "inherited" hypothesis has come from family studies, both at the phenotypic and genotypic level. However the majority of the evidence suggests that the low levels of CR1 are acquired in patients with SLE. This evidence includes data from family studies showing discrepancies between numerical phenotype and genotype; findings of correlations of CR1 levels with indices of disease activity and measures of complement activation; and experiments showing loss of CR1 from erythrocytes transfused into patients with SLE (discussed in chapter 1).

It has previously been observed that there is an association between the presence of antibodies to negatively charged phospholipids, anticardiolipin antibodies, and the presence of positive Coombs' tests in patients with SLE [193]. The finding that reduction of erythrocyte CR1 was associated with disease activity and correlated with erythrocyte C3 and C4 deposition in patients with SLE, autoimmune haemolytic anemia and other conditions characterised by the presence of autoantibodies [175-177], led me to examine the association between anticardiolipin antibodies and reduced levels of CR1 amongst patients with SLE.

Materials and methods

Subjects

Thirty three laboratory personnel and hospital staff were the control panel for this study. Fifty three patients had systemic lupus erythematosus classified by the revised

ARA criteria. Eight patients had the clinical syndrome that has been associated with the presence of anticardiolipin antibodies (16). These subjects all had raised ACA with recurrent spontaneous abortions (n = 3), deep venous thrombosis (n = 3), pulmonary hypertension (n = 1), haemolytic anaemia (n = 2), bone marrow necrosis (n = 1).

Assays for aCL and DNAb

Anticardiolipin antibodies of both IgG and IgM classes were measured on each serum sample by an ELISA method as previously described [226]. Dr S.Loizou kindly performed aCL ELISA assays for this study. Abnormal results were defined as greater than 5 standard deviations above the normal mean value defined in 150 normal subjects (normal IgG < 9.0 aCL ELISA units (AEU), normal IgM <8.0 AEU). Anti-ds DNA antibodies were measured by Farr assay [227] with abnormal results greater than 5 standard deviations above normal mean value. Farr assays were performed by Ms G.Angus with my assistance.

Monoclonal antibodies

The preparation/purification of monoclonal antibody reagents are described in general methods, chapter 2. Monoclonal antibody to CR1 was E11. Antibody to C3d was Clone 3 and to C4d was T2.C5.12. Monoclonal antibodies were radio-iodinated using Iodogen to a measured specific activity, usually between 1 - 2 μCi/μg, as described.

Enumeration of antigens on erythrocytes

The mean number of antigen sites on erythrocytes was measured using a radioligand binding assay as previously described [175] and as detailed in methods: chapter 2.

Assay for C4 in serum

C4 concentration was measured by radioimmunoassay, and are quantified in mg/l by comparison with pool normal serum, calibrated by laser nephelometric estimation of total C4, as described in methods: chapter 2.

Statistical methods

Non-parametric comparisons were performed throughout. Differences between populations were assessed by the Mann-Whitney U test. Correlations were performed by the Spearman Rank test.

Results

6.1 Autoantibodies to cardiolipin and to dsDNA

Levels of aCL were measured amongst the 61 subjects with SLE and with the anti-phospholipid antibody syndrome by ELISA. Twenty seven of these subjects (44%) had levels of IgG or of IgM aCL greater than 5 standard deviations above the normal mean for this assay [226]. DNA binding was greater than 30% in 23 of the 57 (40%) subjects in whom this measurement was obtained. There was no correlation between levels of aCL and of DNAb (Table 15, p 144).

CR1 levels

The mean number of CR1 molecules per erythrocyte amongst the 33 normal subjects was 685 (1 SE = 49), compared with 430 (1 SE = 36) amongst the 34 SLE patients without aCL, and 305 (1 SE = 25) amongst the 27 SLE patients with aCL (Figure 48, below). These values were significantly different between each group.

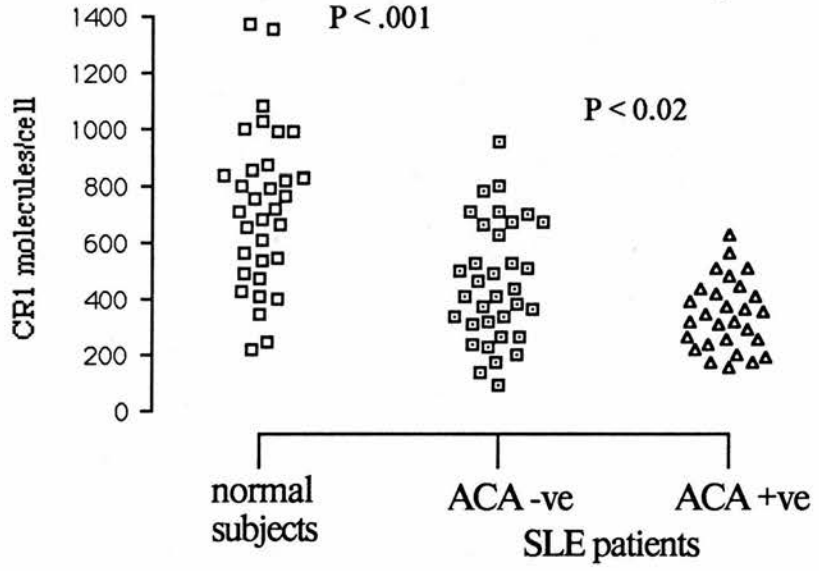

Figure 48. Mean number of CR1 molecules, expressed as molecules per erythrocyte, in 33 normal subjects, 34 SLE patients without anticardiolipin antibodies, and 27 SLE patients with anticardiolipin antibodies. The number of CR1 molecules per red cell amongst the aCL-negative SLE patients was significantly lower than amongst the normal subjects ($z = 3.77$, $p < 0.001$). Similarly the mean CR1 number was significantly lower amongst the aCL-positive SLE patients compared with their aCL-negative counterparts ($z = 2.34$, $p < 0.02$).

6.2 Levels of C4d and of C3d on erythrocytes

The mean number of C4d molecules per erythrocyte amongst the normal subjects was 209 (1 SE = 17), compared with 961 (SE 128) amongst the SLE patients without aCL ($z = 6.5$, $p<0.001$), and 1859 (1 SE = 562) amongst the SLE patients with aCL (aCL- vs aCL+, $z = 1.36$, $p = 0.09$). Similarly the mean number of C3d molecules amongst the normal subjects was 92 (1 SE = 10), amongst the SLE patients without aCL was 308 (1 SE = 51), and amongst the the SLE patients with aCL was 564 (1 SE = 134) (Figure 49). The C3d values were significantly different between each group.

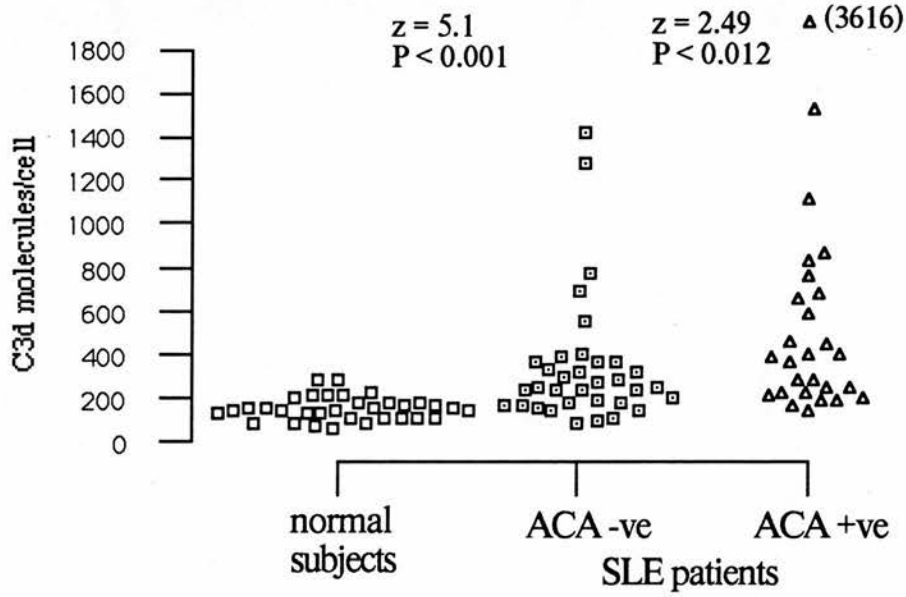

Figure 49: Mean number of C3d molecules, expressed as molecules per erythrocyte, in 33 normal subjects, 34 SLE patients without anticardiolipin antibodies, and 27 SLE patients with anticardiolipin antibodies. The number of C3d molecules per red cell amongst the aCL-negative SLE patients was significantly higher than amongst the normal subjects ($z = 5.1$, $p < 0.001$). Similarly the mean C3d number was significantly higher amongst the aCL-positive SLE patients compared with their aCL-negative counterparts ($z = 2.49$, $p < 0.012$).

6.3 Correlations between aCL levels, CR1 and complement on cells and in serum

The hypothesis was tested that raised levels of aCL were associated with reduced numbers of CR1 and with increased C3d and C4d deposited on erythrocytes. Table 16 (p145) shows correlations between aCL levels (highest value for IgM and IgG) and numbers of erythrocyte CR1, C4d, and C3d, and serum C4. A highly significant negative correlation was observed between aCL levels and CR1 numbers and a highly significant positive correlation between aCL levels and erythrocyte C3d numbers. These correlations remain significant even if corrected for the total number of

correlations performed in this study (n=19). There were only very weak correlations observed between CR1 numbers and erythrocyte C3d and C4d levels (Table 15, below) and these did not alter when the analysis was restricted to those subjects with abnormally elevated deposition of C3d and C4d (> 2SD above the normal mean) on erythrocytes: CR1 versus C4d: RSp = -0.198 (P > 0.05); CR1 versus C3d: RSp = -0.177 (P > 0.05).

Spearman Correlation	n	correlation	probability
aCL			
CR1 number	61	-0.43	**0.001**
erythrocyte C4d	61	0.33	**0.01**
erythrocyte C3d	61	0.41	**0.001**
serum C4	53	-0.09	0.533
DNAb			
CR1 number	57	0.15	0.27
erythrocyte C4d	57	0.08	0.58
erythrocyte C3d	57	0.16	0.24
serum C4	50	-0.43	**0.002**
CR1			
erythrocyte C4d	61	-0.22	0.092
erythrocyte C3d	61	-0.20	0.123
DNAb			
aCL	57	0.33	0.13

Table 15. Correlations between aCL level (highest reading of IgG or IgM), DNAb, and levels of erythrocyte CR1, C4d, C3d, and serum C4 levels.

I then asked whether the effects of the IgM or IgG class of the aCL could be distinguished with respect to CR1 and complement levels on erythrocytes. Both IgG and IgM aCL levels were correlated inversely with CR1 numbers, and with each other (Table 16, overleaf). However, whereas IgG aCL levels showed no correlation with erythrocyte C4d and C3d numbers, highly significant correlations were found between IgM aCL levels and these antigens.

Spearman Correlation	n	correlation	probability
IgG aCL			
CR1 number	61	-0.38	**0.002**
erythrocyte C4d	61	0.12	0.342
erythrocyte C3d	61	0.25	0.051
IgM aCL	61	0.37	**0.004**
IgM aCL			
CR1 number	61	-0.36	**0.004**
erythrocyte C4d	61	0.41	**0.001**
erythrocyte C3d	61	0.40	**0.002**
erythrocyte C4d			
erythrocyte C3d	61	0.73	**< 0.001**

Table 16. Correlations between IgG and IgM aCL levels and erythrocyte CR1, C4d and C3d numbers.

6.4 Correlations between DNAb and CR1 and complement on cells and in serum

The relationships between levels of anti-DNA antibodies and erythrocyte CR1, C4d and C3d and serum C4 concentration were examined in order to test whether the observed correlations between aCL levels and these parameters could simply be explained by an association between the levels of aCL and disease activity. There was no correlation between the levels of aCL and of DNAb (Table 15). Similarly there were no correlations between DNAb and erythrocyte CR1, C4d or C3d (Table 15). There was no correlation between aCL levels and serum C4 levels (Table 15), but in contrast, the DNAb was highly significantly negatively correlated with serum C4 levels (Table 15). This correlation remained significant when corrected for the number of tests performed.

Discussion

The role of erythrocyte CR1 in the processing of circulating ICs and the hypothesis that reduction of CR1 numbers in SLE may contribute to disease susceptibility by reducing the efficiency of protective effector functions of the complement system has been discussed in chapter 1 sections 5 and 6. Observations that CR1 numbers were also low in patients with

autoimmune haemolytic anaemias and in patients with paroxysmal nocturnal haemoglobinuria led to the development of the hypothesis that erythrocytes bearing autoantibodies or opsonic complement fragments may lose their CR1 by receptor proteolysis during the interaction of erythrocytes with cells of the fixed mononuclear phagocytic system [175]. Studies showing that CR1 loss could be demonstrated on erythrocytes transfused into patients with SLE and/or haemolytic anaemias [179] supported the hypothesis that loss of CR1 from erythrocytes occurs within the circulation. Two sets of observations on SLE patients suggested that antiphospholipid antibodies might be a factor associated with reduced CR1 levels on the erythrocytes of patients with SLE. The presence of antiphospholipid antibodies was correlated with positive direct antiglobulin reactions [193]. Low CR1 numbers were similarly associated with positive direct antiglobulin reactions [192]. The authors of this latter study postulated that the link between low CR1 and the positive antiglobulin tests was the presence of immune complexes bound to erythrocyte CR1.

In my studies, a negative correlation (r_S = -0.43) was detected between aCL levels and CR1 numbers on erythrocytes of SLE patients (table 15). This correlation was highly significant despite no account being taken of the subjects' CR1 numerical "genotype" and was not explained by an overall correlation with disease activity since no such relationship existed between aCL and DNAb or serum C4 levels (table 15). The demonstration of erythrocyte complement deposition by the classical pathway could suggest that the relationship between aCL (or a closely related antibody) and low CR1 numbers was the result of direct binding of aCL to the erythrocyte surface. Such direct binding has not yet been unequivocally shown. The strongest evidence has been the observation of aCL activity in the eluate from erythrocytes of a single patient with high serum aCL levels and positive direct antiglobulin reaction [193]. Correlations amongst aCL levels and erythrocyte C4d and C3d levels were found in my studies (table 16). IgM, but not IgG, aCL were found to be correlated highly significantly with the number of C4d and C3d molecules on the patients' erythrocytes. Both IgG and IgM aCL levels correlated inversely with erythrocyte CR1 numbers (table 16), and it is hard to disentangle their relative contributions to the reduction of CR1. However it is noteworthy that there was only a very weak inverse correlation between CR1 numbers and levels of erythrocyte C4d and C3d in the present study. This observation implies that IgG aCL, which were not correlated with levels of complement deposition on erythrocytes, may be related to loss of CR1 by a mechanism independent of complement. An extension of the original hypothesis [175] to incorporate this finding would be that erythrocytes, opsonised with either IgG or complement, lose their CR1 on interaction with cells of the fixed mononuclear phagocytic system. The inclusion of many subjects with IgG aCL in my study may explain the overall

lack of correlation between CR1 levels and C4d and C3d numbers on erythrocytes, differing from previous observations [175].

In contrast to the studies of Norberg and colleagues [228] and Hazeltine and coworkers [193], I found no association between aCL and reduced levels of serum C4 (table 15), although I did find the previously described inverse correlation between levels of anti-dsDNA antibodies and serum C4 levels [229]. This may be explained by our studying different populations. Norberg and colleagues [228] compared C4 levels between patients with aCL and recurrent spontaneous abortions and women with recurrent spontaneous abortions and no antiphospholipid antibodies. The population of patients with aCL studied here comprised mainly patients with SLE, amongst whom many other autoantibodies which fix complement were present, eg anti-dsDNA antibodies. However, the strength of the negative correlation (-0.43) between the level of aCL and mean erythrocyte CR1 numbers suggests that aCL may be an important acquired factor determining the reduction of erythrocyte CR1 in patients with SLE.

Results

Chapter 7

Antiphospholipid antibody erythrocyte binding

In patients with SLE [194,230] or the 1°APL syndrome [231], the presence of antiphospholipid (aPL) antibodies is associated with haemocytopaenia. In addition, cardiolipin binding reactivity has been eluted from the Coombs' positive erythrocytes of patients with SLE [193]. These observations, along with the evidence presented in the preceding chapter, suggest that aPL bind directly to erythrocytes in patients with connective tissue diseases and raise the possibility that aPL are a common anti-erythrocyte (and anti-platelet) antibody. I therefore surveyed the prevalence of aCL positivity amongst patients selected on the basis of a positive Coombs' test, and attempted to demonstrate direct binding of polyclonal and monoclonal aCL antibodies to normal, SLE and Coombs' positive erythrocytes.

Cardiolipin binding antibodies in patients with SLE show cross reactivity with negatively charged phospholipids (PLs) such as phosphatidylcholine, but do not commonly binding to neutral PLs such as phosphatidylserine [232]. The erythrocyte membrane is asymmetric [233]. The external lipid bilayer consists mainly of neutral moieties and normally expresses only low levels of negatively charged PLs, though there is a degree of mobility (flip-flop) of lipids between the two leaflets [233]. I therefore tested the hypotheses that aPL antibodies in patients with AIHA showed increased binding to neutral PLs and that manipulation of the erythrocyte surface to expose more negatively charged PLs would alter aPL binding.

Patients, Materials and Methods

Serum and erythrocyte samples were collected from thirty patients attending the haematology department, Hammersmith Hospital for the assessment of a positive Coombs' test, or in the follow-up of known Coombs' positive haemolytic anaemia. Erythrocytes were washed 3 times in PBS/BSA buffer and stored at 4°C, while serum samples were separated and stored at -70°C usually on the day of venesection, as described.

Cell surface C3, C4 and CR1 numbers were enumerated by radioligand binding in the usual manner. Serum cardiolipin binding was kindly measured by Dr S. Loizou by ELISA assay as previously described [226]. IgG and IgM antibody binding to a panel of phospholipid (PL) antigens was measured by an adaptation of this method in which, 5 other negatively charged and 4 neutral PLs were used as the solid phase antigen in place of

cardiolipin in ELISA assay [232]. 16 of these Coombs' positive subjects were tested for cross-reactive PL binding in this way. Eight of these were known to have been aCL positive, and 8 had never shown reactivity to cardiolipin.

Antibodies and radiolabelling

Anti-CR1 (E11), anti-C3d (clone 3) and anti-C4d (T2, C5.12) and Iodogen labelling techniques were used as described above. The IgM monoclonal antibody BH1 was derived from the lymphocytes of a patient with the 1° APL syndrome [199]. The patient, BH has been reported elsewhere (previously referred to as patient 9 [234]). The complications suffered by this patient include 4 first or second trimester abortions, 8 episodes of visual loss, mild thrombocytopaenia, livedo reticularis, Raynauds' phenomenon and aortic regurgitation. The patients' serum and BH1 were known to bind to cardiolipin and to be positive in the lupus anti-coagulant test, but to be VDRL negative and to show no cross-reactivity with dsDNA. BH1 is thus thought to be typical of aPL antibodies. Furthermore, BH1 is known to bind to all of the panel of negatively charged PLs used in these experiments, though not to neutral PLs and is able to inhibit the binding of IgG class aCL to cardiolipin, implying similar binding specificity. Polyclonal IgG and IgM class aCL antibodies were purified from high titre cardiolipin binding patient sera by elution from cardiolipin liposomes and were kindly donated by Dr S.Loizou. Immunoelectrophoresis of these antibodies confirmed that the IgG preparation was free of IgM and vice-versa.

Radiolabelling of aCL antibodies proved to be difficult and initial attempts to label polyclonal aCL by the Iodogen method led to inactivation of cardiolipin binding, though BH1 could be successfully labelled by this method. Polyclonal IgG and IgM aCL were therefore labelled by the lactoperoxidase (Sigma Pharmaceuticals) method described by Thorel and Johansson [235] with minor modifications. Briefly, lactoperoxidase was incubated with antibody at at 0.8μg/μg antibody for 5 minutes at 22°C in the presence of 0.88% H_2O_2 after careful exclusion of NaN3 from all reagents. Bound label was then separated from free $Na^{125}I$ and the specific activity and antibody concentration calculated by TCA precipitation as described. Specific reactivities of 2-4 x 10^6/μg were achieved for BH1, but lower specific activities (2.5 x 10^5cpm/μg) were acheived with polyclonal IgG and IgM aCL. Functional integrity of the labelled antibody was demonstrated by the finding of specific binding of 5.4% of offered IgG, 2.1% of IgM and 26% of labelled BH1 to ACA in ELISA wells, using aCL blocked with 2% BSA as control..

Results

7.1 Erythrocyte C3, C4 and CR1 amongst Coombs' positive subjects

Mean erythrocyte C3d was 886.3 molecules/cell (+/-307.3, 1SE) and C4d was 2199.5 (+/-496.5) amongst 22 of these patients, while mean CR1 numbers were 590.4 (+/-59.2). For comparison mean C3d, C4d and CR1 amongst 33 normal control subjects were 92 (+/-10), 209 (+/-17) and 605 (+/- 49), respectively. While C3 and C4 numbers are raised, CR1 numbers amongst these subjects were not significantly different from the normal population (U = 277.5, P = 0.07).

7.2 The Prevalence of aCL Antibodies amongst Coombs' positive subjects

Nine of these 30 AIHA patients (30%) showed positive aCL titres, 8 IgM and 1 IgG. The clinical details and aCL levels amongst these patients is shown below in table 17. Allowing that aCL may be primarily associated with SLE in the 2 patients with this diagnosis, the prevalence of aCL amongst Coombs' positive patients was at least 25.0%, compared with the normal population (<2%, Dr S.Loizou, personal communication).

Autoimmune neutropenia (2)		
patient 1	female aged 88	IgG ACA 6.4: IgM ACA **27.0**
patient 2	female aged 66	IgG ACA 1.7: IgM ACA **12.0**
kerato-conjunctivitis sicca Coombs' positive for complement only		
Haematological malignancy (2)		
patient 1	CLL	IgG ACA **7.0**: IgM ACA **22.0**
patient 2	T cell lymphoma	IgG ACA 6.4: IgM ACA **8.0**
(AIHA and autoimmune thrombocytopenia)		
Coombs' positive (1)		
coincident finding		IgG ACA 1.2: IgM ACA **27.0**
AIHA (2)		
patient 1	severe intractable AIHA	
		IgG ACA 0.8: IgM ACA **168.0**
patient 2		IgG ACA 6.5: IgM ACA **64.0**
SLE (2)		
patient 1	AIHA 8 yrs prior to SLE	
		IgG ACA 1.0: IgM ACA **62.0**
patient 2		IgG ACA **9.0**:IgM ACA **34.0**

Table 17. Prevalence of aCL antibodies amongst 30 Coombs' positive patients.

7.3 Cross reactivity with negative and neutral phospholipids amongst Coombs' positive subjects

IgG and IgM cross-reactivity of 16 Coombs' positive sera against a panel of neutral and negatively charged PLs was tested by Dr S.Loizou. Negatively charged PLs used were: cardiolipin (CL), phosphatidic acid (PA), phosphatidyl inositol (PI), phosphatidyl glycerol (PG) and phosphatidyl serine (PS). Neutral PL were phosphatidyl ethanolamine (PE), phosphatidyl choline (PC), phosphatidyl l-α phosphatidyl choline, γ-acetyl-ß-*O*-alkyl (platelet activating factor, PAF) and Sphingomyelin (SPH). Half of these subjects were known aCL positive, and half were aCL negative. A panel of 8 normal control sera were used. Mean (+/-1 SD) IgM PL binding was 0.384 (+/- 0.593) AEU, while IgG controls showed 0.436 (+/- 0.685) AEU. Significant binding was defined as > 5 SD above the mean of 8 normal controls as before [226]. Figure 50: A and B (overleaf) shows the results of these measurements expressed as SD *above* the mean of normal control binding, the normal range is shown cross-hatched. Ten individuals were positive in these assays and extensive cross-reactivity was noted. For IgM, 10 (62.5%) showed binding to negatively charged PLs, 8 of whom were reactive to cardiolipin and 2 (12.5%) showed -ve PL reactivity independent of CL binding. Eight subjects showed IgM binding to neutral PLs (50%), 7 of whom were cross reactive with CL, the remaining individual was positive for sphingomyelin binding only. With regard to IgG, 7 subjects (43.8%) were positive and all bound to -ve PLs. Six (37.5%) showed CL binding and 1 was independent. Four (25%) individuals showed IgG binding to neutral PLs, 3 cross reactive with CL and 1 independent of this specificity. As indicated, cross reactivity amongst these sera was extensive. The results of Spearman rank correlation between levels of IgG and IgM aCL antibodies and antibodies to other PLs is shown below in table 18.

		Negative				**Neutral**			
		PA	**PG**	**PI**	**PS**	**PC**	**PE**	**PAF**	**SPH**
IgG aCL	rSp	0.65	0.72	0.88	0.69	0.36	0.66	0.54	0.63
	P<	0.006	0.002	0.001	0.003	**0.166**	0.005	0.032	0.009
IgM aCL	rSp	0.92	0.79	0.98	0.88	0.65	0.56	0.75	0.86
	P<	0.001	0.001	0.001	0.003	0.006	0.025	0.001	0.001

Table 18: Correlation of IgG and IgM CL binding with binding to negative and neutral PLs amongst 16 Coombs' positive subjects. Non-significant correlations are shown in bold.

Figure 50A and 50B. Binding of IgM and IgG aPL antibodies to negatively charged and neutral phospholipids amongst 16 Coombs' positive subjects

Figure 50 A

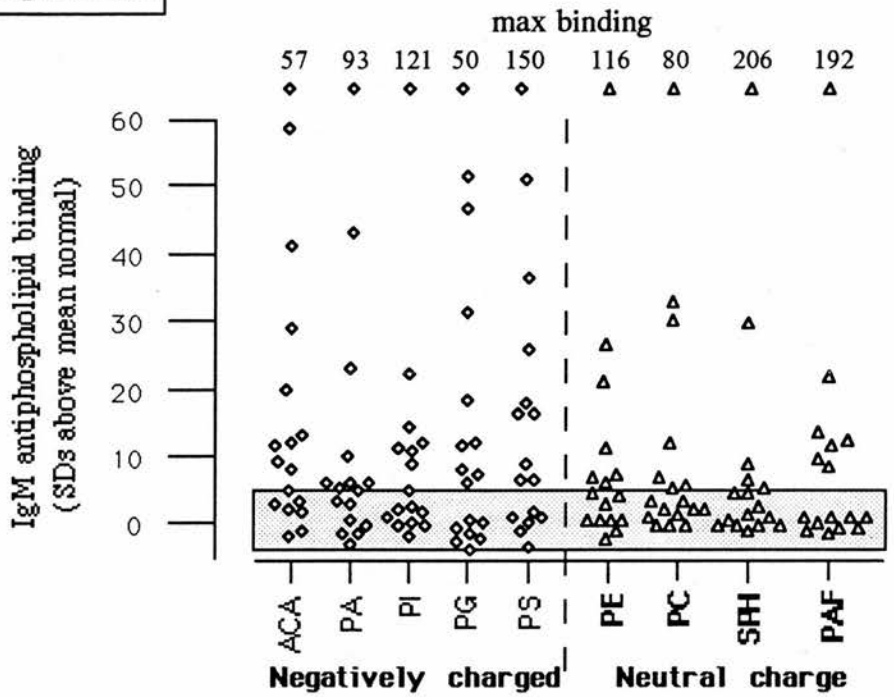

Figure 50 B

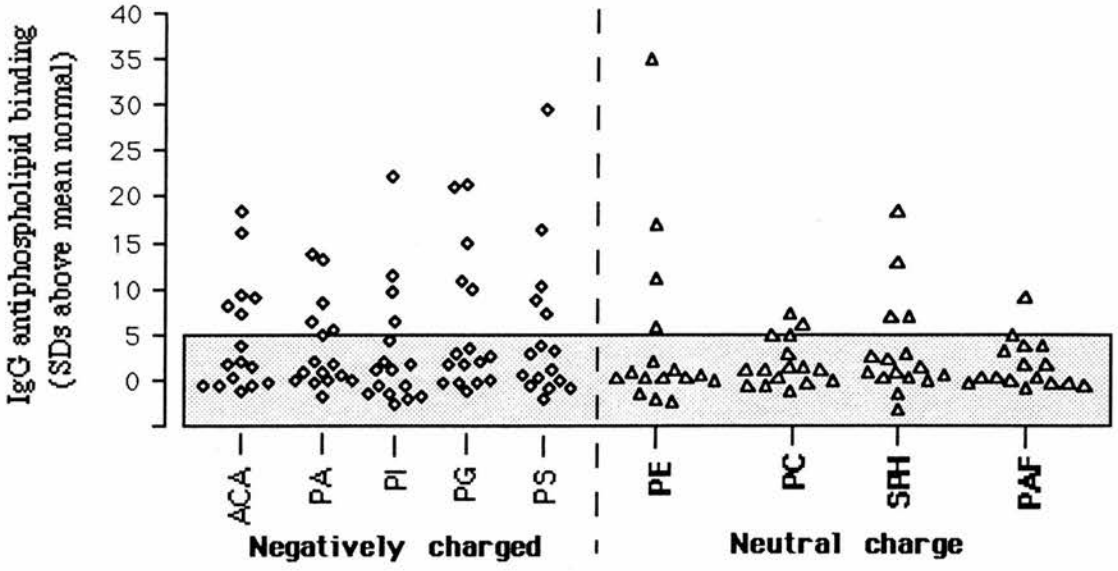

Figure 50A and B. Binding of IgM and IgG aPL antibodies to negatively charged and neutral phospholipids amongst 16 Coombs' positive subjects. The normal range (<5 SD above the mean of normal binding) is indicated by the cross-hatched area.

7.4 Ligand Binding of Purified aPL Antibodies

To investigate the potential cellular pathogenicity of aPL antibodies an attempt was made to demonstrate the binding of purified aPL antibodies to erythrocytes. The purified IgM monoclonal antibody BH1 is described above as are patient derived affinity purified polyclonal IgG and IgM aCL binding antibodies which were kindly donated by Dr S.Loizou.

7.41 Experiments with BH1

A number of experiments were performed and will be described in outline since no evidence of BH1 binding to erythrocytes could be demonstrated. BH1 was radiolabelled by Iodogen and by the lactoperoxidase method with minor modifications. The following experiments were then performed.

Direct Binding of BH1 to Erythrocytes

BH1 was incubated with erythrocytes from normal control subjects (n = 6), patients with SLE (n = 11) and Coombs' positive, non SLE subjects (n = 5) under the same conditions as those employed for radioligand binding studies. Mean percentage BH1 bound was 0.63, 0.71, and 0.74 respectively. When serial dilutions of radiolabelled BH1 were incubated with known numbers of erythrocytes from these 3 study groups, no evidence of a specific binding effect could be detected. Similarly, when serial dilutions of erythrocytes were incubated with a potentially saturating concentration of labelled antibody (15μg/ml), a consistent level of apparent binding suggestive of non-specific background only (0.5%) was found. The same results were obtained with incubation at 37, 22 and 4°C regardless of duration up to 18hrs.

Indirect Evidence of BH1 Binding to Erythrocytes

The possibility that weak BH1 binding was occurring followed by elution during experimental procedures was tested by examining normal (n=2) and SLE (n=2) erythrocytes for evidence of complement deposition subsequent to incubation with unlabelled BH1 and autologous serum using EDTA and no-antibody controls, at differing temperatures as above. No evidence of deposition of C3, C4 or reduction of CR1 could be found.

Binding of BH1 to Erythrocyte Ghosts

Since the distribution of erythrocyte membrane lipids is asymmetric and negative PLs are mainly internal, the possibility that BH1 may binding to internal membrane lipids was tested by incubation of radiolabelled BH1 to erythrocyte ghosts prepared by sonication of normal erythrocytes. No specific binding could be found.

7.42 Studies with Affinity Purified polyclonal IgG and IgM aCL

Binding of ^{125}I aCL to Erythrocytes and ^{125}I Protein A probing

Serial dilutions of radiolabelled IgG and IgM antibodies were incubated with normal human erythrocytes using the method described for radioligand binding studies. No evidence of specific binding could be detected. To exclude the possibility that radiolabelling damage was affecting the reagents, serial dilutions of 100µl unlabelled IgG aCL starting concentration 100µg/ml were incubated with 100µl normal human erythrocytes at 1.5 x 10^8/ml PBS/BSA buffer, using irrelevant human IgG as control. These were then washed 3 times to remove unbound antibody and probed with ^{125}I Staphylococcal Protein A (1µg, 10^5 cpm) prior to separation of bound antibody by centrifugation through oil. The results are shown below in figure 51 and suggest that some specific binding of IgG aCL was present. This did not reach saturation.

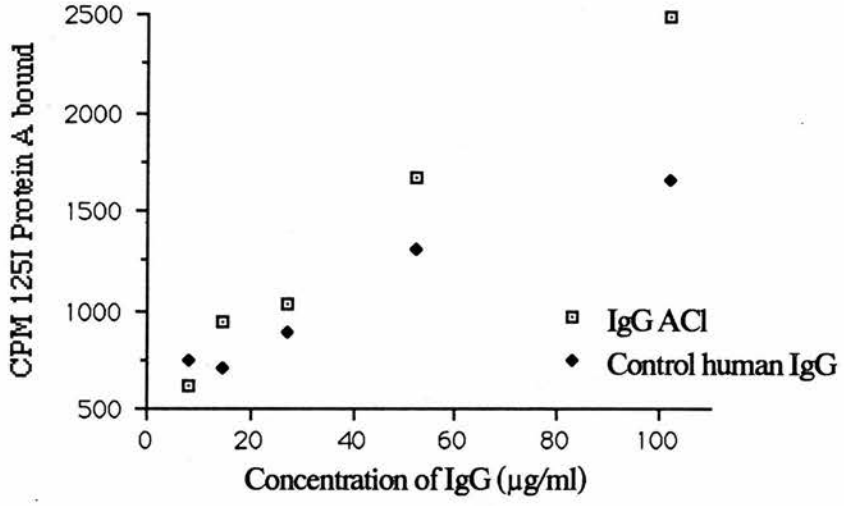

Figure 51. Binding of ^{125}I labelled Staphylococcal Protein A to normal human erythrocytes incubated with affinity purified IgG aCL and with irrelevant human IgG control.

Binding of ^{125}I aCL to altered erythrocytes

Sodium Tetrathionate is a cellular metabolic inhibitor which has been shown to induce a glucose dependent alteration in erythrocyte cell membrane lipid composition [236]. Incubation of cells with tetrathionate has been shown to increase the exposure of phosphatidylethanolamine (a negatively charged PL) from 5 to 20% . These changes are antagonised by pre-incubation with glucose. In view of the failure of ^{125}I IgM aCL, and the indirect evidence of IgG binding to native erythrocyte membranes, the binding of radiolabelled IgG, IgM aCL and BH1 to Na Tetrathionate treated erythrocytes was tested.

Materials

Na Tetrathionate (Sigma pharmaceuticals)

Incubation buffer: NaCL 50mM, KCl 100mM, Na_2HPO_4/NaH_2PO_4 10mM, sucrose 44mM. pH 7.4

Method

Tetrathionate treatment of erythrocytes

Washed normal human erythrocytes were incubated in 10 volumes of incubation buffer with 11mM glucose added to control cells for 24 hrs at 37°C with gentle rotation. 5mM NA tetrathionate was then added, the pH adjusted to 8.0 and incubation continued at 4°C for 4hrs.

^{125}I aCL probing

Cells were washed 3 times with PBS/BSA buffer, adjusted to at 5 x 10^8/ml, incubated with ^{125}I antibody (5µg/ml) prior to separation of bound from free by centrifugation of duplicate 125µl aliquots through oil as before. Conditions were adjusted so that each 125µl aliquot contained approximately 3.5 x 10^5cpm labelled antibody. Cells were probed with ^{125}I:IgG and IgM aCL and BH1. E11 was used as a control for non-specific, proteolytic removal of cell surface antigens. Results are shown in figure 52. By comparison, treatment of erythrocytes with papain caused proteolytic loss of cell surface receptor and reduced E11 binding by 41.5%.

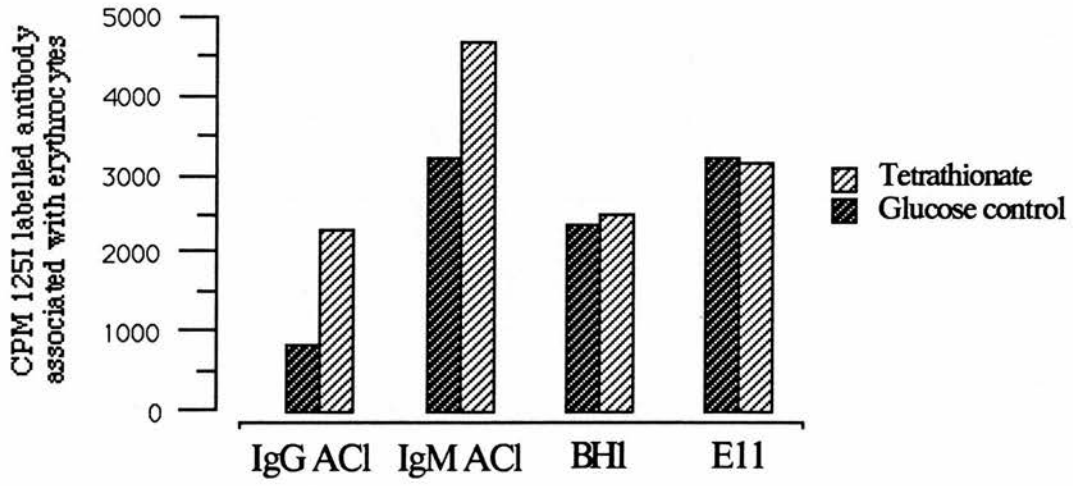

Figure 52. Binding of radiolabelled aPL antibodies and control (mab E11) to human erythrocytes treated with Na Tetrathionate in the presence or absence of glucose (control).

Discussion

Anticardiolipin antibodies are a commonly occurring member of a family of antibodies which bind principally to the phosphodiester group of negatively charged PLs. Clinically, aCL are associated with prolongation of coagulation tests *in vitro* (the lupus anticoagulant test) but venous and arterial thromboses *in vivo*, recurrent abortion, livedo reticularis, neurological disease and also thrombocytopaenia and Coombs' positivity (see [237] for review). These antibodies may occur as a solitary immunological abnormality (the primary antiphospholipid syndrome), or as part of a connective tissue disease such as SLE, where their presence appears to define a subgroup of patients characterised by haemocytopaenias [194]. Deleze and colleagues [238] studied 500 patients with SLE and concluded that IgG aCL were associated with thrombocytopaenia, while IgM aCL were associated with haemolytic anemia and a positive Coombs' test. Direct binding of aCL to native blood cells has been difficult to demonstrate and typically has required extensive alteration to the cell surface [239], though binding to erythrocytes has been inferred [193] and binding to platelets has been reported [240].

Cardiolipin (diphosphatidylglycerol) is a common antigen, however, its location in the mitochondrial membrane [241] makes it unlikely to be an antigen directly responsible for erythrocyte cell surface binding. The external bilayer of the erythrocyte membrane comprises mainly of the neutral PLs phosphatidylcholine (PC) and sphingomyelin (SPH), while the internal leaflet contains both phosphatidylethanolamine (PE, neutral) and phosphatidylserine (PS, negatively charged) [242], though a degree of crossover (flip-flop) of internal PS to the exterior of the cell does occur [233]. Anti-cardiolipin antibodies show extensive cross-reactivity with other negatively charged PLs [232], but little binding to neutral PLs. It seems likely, therefore, that for significant erythrocyte binding to occur, the cell membrane must be abnormal in its lipid composition, as is the case with sickle-cells [243] or that erythrocyte-binding antibodies are a subset with a high degree of cross-reactivity to neutral PLs. Cabral and colleagues have recently described the isolation of an IgM class aCL antibody from a patient with HA (but not SLE) which showed binding to phosphatidylcholine (neutral) and to bromelain treated erythrocytes [244] and that PC cross-reactivity was more common in aCL positive SLE subjects with haemolytic anemia than those without this complication . The studies described here show that aCL antibodies are common in Coombs' positive subjects without connective tissue diseases, being present in 7 of 28 (25%) subjects, and in a further 2 (making a total of 9 out of 30 subjects surveyed) with concommitant SLE, giving an overall prevalence of 30%. This suggests that aCL may be a common anti-erythrocyte autoantibody in non connective tissue disease subjects [196].

Sixteen of these Coombs' positive subjects were then tested for cross-reactivity against a panel of negative and neutral PLs. Previous studies [232] have demonstrated the heterogeneity of lipid binding and functional characteristics amongst aCl and aPL antibodies from patients with SLE and syphilis. The results obtained with these Coombs' positive non SLE patients may suggest further heterogeneity amongst disease groups and indicate an increase in binding to neutral PLs which may be of functional significance. In the study of Loizou and colleagues [232] subjects with SLE showed low levels of cross-reactivity with neutral PLs (27.3% for IgM and 16.4% for IgG antibodies, Dr S.Loizou, personal communication), while neutral PL binding was almost as common as negative PL binding amongst subjects with syphilis. Amongst these Coombs' positive subjects, 50% showed IgM and 25% IgG binding to neutral PLs. As shown in table 18, both IgG and IgM neutral PL-binding antibodies showed significant cross-reactivity with CL as judged by Spearman correlation analysis. Amongst the patients reported by Loizou et al, significant correlations with CL amongst IgG antibodies were typical of syphilis not SLE subjects. Moreover the dominant cross-reactive neutral PL target amongst SLE patients in Loizou's study was PC, while the correlation between PC and CL amongst Coombs' positive subjects was not significant (table 18). Antiphospholipid antibodies in Coombs' positive subjects thus seem to resemble those in patients with syphilis more than SLE, but differ in being associated with the haemocytopaenias which are typical of the connective tissue diseases. However, I consider these to be preliminary findings only, since the number of Coombs' positive patients were small and the selection of equal numbers of aCL positive and negative patients may have biased the results. Against selection bias significantly affecting these results is the fact that in the study of Loizou et al IgG class neutral PL binding was found in 18.5% of 27 CL positive and 14.3% of 28 CL negative SLE subjects. A mean of 16.4% would thus be expected in a balanced sample of aCL positive and aCL negative subjects, compared with 25% binding found amongst Coombs' positive subjects.

Clearly, the property of neutral PL binding may be important in binding to the erythrocyte membrane since the external leaflet is primarily composed of the neutral moieties. Alternatively, the low levels of negatively charged PL (-ve PL) present on the external surface of normal E may be the antigenic target for the commoner -ve PL binding antibodies. I attempted to study this latter possibility directly using a monoclonal antibody reactive with -ve PLs and polyclonal aCL antibodies affinity purified by binding with -ve PL liposomes. The monoclonal antibody BH1 showed no evidence of direct erythrocyte binding despite extensive testing. BH, from whom this antibody was isolated had suffered thrombocytopaenia and complications typical of the 1° APL syndrome, but not haemolytic anaemia. Further experiments with both IgG and IgM polyclonal aCL support

the hypothesis that aCL may bind directly to erythrocytes (figure 51), though this has not been exhaustively proven. The finding that polyclonal aCL binding is increased by treatment of red cells with Na Tetrathionate (figure 52) suggests that it is the lipid-binding properties of the antibody which determines binding. Na Tetrathionate oxidises SH groups and is capable of exposing the *internal* -ve PL, phosphatidylserine, to digestion by exocellular pancreatic phospholipase C [236] in whole erythrocytes, suggesting that treatment with methionine induces a reorientation of PLs between the internal and external lipid bilayers. It is also of note that quantitatively the binding of aCL in this experiment is equivalent to that of E11 which recognises a specific cell surface ligand.

Conclusions

The aim of this work was to study factors which may contribute to disease expression in SLE by impairing the efficiency of complement- and erythrocyte CR1-mediated mechanisms of immune complex clearance, specifically inherited partial deficiency of C4A and acquired reduction of erythrocyte CR1 expression.

Initial experiments employed a newly developed radioimmunoassay to measure serum concentrations of C4 isotypes separately and were intended to contribute to understanding the physiology of C4, specifically the complex relationship between C4 genotype and phenotypic expression. Since serum levels of C4 reflect both production and consumption genotyped normal sera in which an acute phase response was excluded were employed. I then investigated the pathophysiology of C4 in SLE by studying C4 immune complex binding in two models chosen to highlight the differences between C4A and C4B and to reflect some of the complexities of *in vivo* immune complex processing. To conduct these studies I developed a second new assay for the quantification of C4A and C4B on HAGG immune complexes.

After this, I considered factors contributing to reduction of erythrocyte CR1 numbers, which may also reduce the efficiency of IC clearance in subjects with SLE. Specifically, I investigated the association of aCL antibodies with erythrocyte CR1 reduction and pursued the physiological link underlying my findings by investigating the occurrence and phospholipid binding of aCL in other conditions characterised by a positive Coombs' test and by experiments with purified antiphospholipid antibodies.

The principal findings of these studies have been:

1. The difficulty in identifying C4 genotype from phenotype has been confirmed in these studies and a number of factors relating to C4 expression have been explored including age, gender, expression of homo-duplicated C4A genes and anomalous antigenic recognition by anti-C4 monoclonals. I have confirmed that the C4B null allele encoded in the extended haplotype linked with Felty's syndrome is associated with an expressed homoduplicated C4A gene, making . The possibility that C4 isotypes respond differentialy to acute phase stimuli was not resolved by the study undertaken here.

2. That an excess of C4B is found on SLE erythrocytes *in vivo*, confirming previous findings *in vitro*.

3. That C4B may form a more efficient erythrocyte surface C3 convertase than C4A

4. That the relationship between C4 isotype levels and opsonisation of protein ICs is more complex in whole serum than is apparent when purified C4A and C4B are used *in vitro.* While the predicted predominance of C4A binding was not confirmed in these studies, the finding that C4A levels correlate more closely with IC deposition may suggest a subtle difference in the efficiency of C4 isotype opsonisation of ICs of protein antigens.

5. That aCL antibodies contribute to reduced CR1 expression in patients with SLE, and that IgM aCL are associated with erythrocyte complement deposition.

6. That aCL antibodies may be a common anti-erythrocyte autoantibody in Coombs' positive patients

7. That aPL antibodies in Coombs' positive patients shows increased cross-reactivity with neutral PLs, which are likely to be important antigens on the external erythrocyte membrane

8. Some evidence was found that polyclonal aCL antibodies can bind directly to erythrocytes and that modulation of the erythrocyte surface lipid composition increases this, providing further mechanistic support for the association of aCL with CR1 reduction.

The hypothesis summarising these data and linking the association of aCL with E-CR1 reduction is that a subset of the heterogeneous population of aPL immunoglobulins are common anti-erythrocyte autoantibodies. These E-binding antibodies are cross-reactive with CL and show increased binding to neutral PLs, a common erythrocyte surface antigen. Alternatively, although negatively charged PLs comprise a small proportion of the external erythrocyte membrane, IgG antibodies with this specificity are a frequent finding and could be quantitatively important. IgM antibodies are associated with erythrocyte complement fixation, opsonising the cell for interaction with complement-receptor bearing phagocytes of the MPS and resulting in secondary reduction of E-CR1. The mechanism linking IgG antibodies appears to be C-independent but could involve phagocyte Fc receptors.

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Appendix

Publications arising from this thesis

Hammond, A., AC. Rudge, S. Loizou, SJ. Bowcock, and MJ. Walport. 1989. Reduced numbers of CR1 on erythrocytes from patients with SLE and the antiphospholipid syndrome are associated with increased levels of anticardiolipin antibodies. Arthritis and Rheumatism, 32:259-264.

Hammond, A., W. Ollier, A. Silman, and MJ. Walport. 1991. Effects of C4 null alleles and homoduplications on the quantitative expression of C4A and C4B. In submission.

Mackworth-Young, C.G., S. Loizou, PK. Thompson, C. Cofiner, A. Hammond, and MJ. Walport. 1991. A human monoclonal antiphospholipid antibody. In submission.

Walport, M.J., Hammond,A., Davies, K.A.A., Moldenuaer, F. 1989 Complement receptor type 1 and SLE. In Proceedings of the Second International Conference on SLE. Professional Postgraduate Services, Tokyo, 52-55.

Publications in abstract

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Hammond A, Rudge AC, Loizou S, Bowcock SJ, Walport MJ. 1988. Anticardiolipin antibodies (ACA), complement, and erythrocyte complement receptor type 1 (CR1). British Journal of Rheumatology. 27:2. 151 (S).

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Reduced numbers of CR1 on erythrocytes from patients with SLE and the antiphospholipid syndrome are associated with increased levels of anticardiolipin antibodies

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Abstract

The numerical expression of complement receptor type 1 (CR1) is reduced on erythrocytes of patients with several diseases including SLE and autoimmune haemolytic anaemias. Amongst patients with SLE, anticardiolipin antibodies (ACA) have been associated with positive direct antiglobulin tests. Because of these findings, we asked whether the reduced erythrocyte CR1 numbers amongst 53 patients with SLE and 8 with the "antiphospholipid syndrome" might be associated with the presence of ACA. A negative correlation was observed between ACA levels and mean CR1 numbers (RSp = -0.43, p =0.001) and a positive correlation between ACA levels and the levels of erythrocyte C4d and C3d (RSp = 0.33 and 0.41, p = 0.01 and 0.001 respectively), but no correlation of ACA levels with serum C4 levels. When the results were further analysed according to the IgG or IgM class of ACA, levels of antibodies of both classes were negatively correlated with CR1 numbers, but only IgM ACA levels were correlated with erythrocyte C4d and C3d numbers. The levels of anti-dsDNA antibodies showed no correlation with erythrocyte CR1, C4d or C3d numbers but were negatively correlated with serum C4 levels (RSp = -0.43, p = 0.002). These data suggest that ACA, or a closely related antibody specificity, may bind to erythrocytes and be directly involved in the mechanism for reduction of erythrocyte CR1 numbers amongst SLE patients.

Introduction

Primate erythrocytes bear a receptor for C3b, iC3b and C4b, named complement receptor type 1 (CR1, CD35), which is believed to have two main physiological activities on these cells (reviewed in 1). CR1 functions as a cofactor to the serine esterase, Factor I, in the catabolism of C3b to iC3b, and of iC3b to C3c and C3dg. It may also bind immune complexes and micro-organisms which bear fixed C3b, iC3b or C4b, and transport them through the circulation to the fixed mono-nuclear phagocytic system (reviewed in 2).

CR1 displays a numerical polymorphism on erythrocytes which is controlled by both inherited and acquired factors. Amongst normal humans, the number of CR1 molecules on erythrocytes varies between means of approximately 100 and 1500 molecules per cell (reviewed in 3). The level of numerical expression is stable amongst normal subjects and is, in part, regulated by a locus encoded in close vicinity to the CR1 structural gene on Chromosome 1 (4, 5). It has been shown that CR1 numbers are reduced on the erythrocytes of patients with several diseases including SLE (6, 7, 8, 9) autoimmune haemolytic anaemias (10), paroxysmal nocturnal haemoglobinuria (11), AIDS (12), and lepromatous leprosy (13). There has been dispute whether the mechanism for this reduction amongst SLE patients is inherited or acquired (reviewed in 3, 14). Evidence for the "inherited" hypothesis has come from family studies, both at the phenotypic and genotypic level. However the majority of the evidence suggests that the low levels of CR1 are acquired in patients with SLE. This evidence includes data from family studies showing discrepancies between numerical phenotype and genotype; findings of correlations of CR1 levels with indices of disease activity and measures of complement activation; and experiments showing loss of CR1 from erythrocytes transfused into patients with SLE.

It has previously been observed that there is an association between the presence of

antibodies to negatively charged phospholipids, anticardiolipin antibodies, and the presence of positive Coombs' tests in patients with SLE (15). In view of the finding that patients with autoimmune haemolytic anaemias have low erythrocyte CR1 levels (10), this led us to examine the association between anticardiolipin antibodies and reduced levels of CR1 amongst patients with SLE. Our results show an association between the presence of anticardiolipin antibodies and reduced levels of CR1 on erythrocytes.

Materials and methods

Subjects

Thirty three laboratory personnel and hospital staff were the control panel for this study. Fifty three patients had systemic lupus erythematosus classified by the revised ARA criteria. Eight patients had the clinical syndrome that has been associated with the presence of anticardiolipin antibodies (16). These subjects all had raised ACA with recurrent spontaneous abortions (n = 3), deep venous thrombosis (n = 3), pulmonary hypertension (n = 1), haemolytic anaemia (n = 2), bone marrow necrosis (n = 1).

Assays for ACA and DNAb

Anticardiolipin antibodies of both IgG and IgM classes were measured on each serum sample by an ELISA method as previously described (17). Abnormal results were defined as greater than 5 standard deviations above the normal mean value defined in 150 normal subjects (normal IgG < 9.0 ACA ELISA units (AEU), normal IgM <8.0 AEU). Anti-ds DNA antibodies were measured by Farr assay (18) with abnormal results greater than 5 standard deviations above normal mean value.

Monoclonal antibodies

Monoclonal antibody to CR1 was E11 (19, donated by Dr N Hogg, ICRF, Lincoln's Inn Fields, London). Antibody to C3d was Clone 3 (20, donated by Prof P Lachmann, MRC Centre, Cambridge) and to C4d was T2.C5.12 (donated by Prof G Ross, Chapel

Hill, N Carolina). Monoclonal antibodies were radio-iodinated using Iodogen (21) to a measured specific activity, usually between 1 - 2 μCi/μg.

Enumeration of antigens on erythrocytes

The mean number of antigen sites on erythrocytes was measured using a radioligand binding assay as previously described (9). All of the radiolabelled antibodies were incubated at a final concentration of 5 μg / ml with erythrocytes suspended in PBS / 1% BSA/ 10mM NaN_3 at 3.3 x 10^8 / ml. Antigen sites were greater than 95% saturated with antibody under these conditions.

Assay for C4 in serum

C4 concentration was measured by radioimmunossay. All incubations were performed in PBS / NaN_3 10mM / Tween 20 0.05% / BSA 0.2% / EDTA 10mM pH 7.2 in polystyrene microtitre plates (Immulon II Removawells, Dynatech, Billingshurst, Sussex). These plates were coated at 10 μg / ml for 24 hours at 4°C with rabbit anti-human C4 (Serotec, Kidlington, Oxford) which had been affinity-purified on C4-Sepharose CL4B (gift of Dr R A Harrison, MRC Centre, Cambridge) and subsequently blocked using 6 washes of buffer. Test sera were diluted to a concentration of 1:1640 and 50μl samples were incubated in duplicate in wells for 4 hours at 37°C, washed 6 times, incubated with 50μl of murine monoclonal anti-C4 at 2 μg / ml, washed 6 times, and probed with 50μl of ^{125}I-affinity-purified rabbit anti-mouse IgG (100,000 dpm / well, approximately 2 μg / ml). Results were expressed as % of the C4 concentration in a pool of 20 normal sera.

Statistical methods

Non-parametric comparisons were performed throughout. Differences between populations were assessed by the Mann-Whitney U test. Correlations were performed by the Spearman Rank test.

Results

Autoantibodies to cardiolipin and to dsDNA

Levels of ACA were measured amongst the 61 subjects with SLE and with the anti-phospholipid antibody syndrome by ELISA. Twenty seven of these subjects (44%) had levels of IgG or of IgM ACA greater than 5 standard deviations above the normal mean for this assay (17). DNA binding was greater than 30% in 23 of the 57 (40%) subjects in whom this measurement was obtained. There was no correlation between levels of ACA and of DNAb (Table 1).

CR1 levels

The mean number of CR1 molecules per erythrocyte amongst the 33 normal subjects was 685 (1 SE = 49), compared with 430 (1 SE = 36) amongst the 34 SLE patients without ACA, and 305 (1 SE = 25) amongst the 27 SLE patients with ACA (Figure 1). These values were significantly different between each group (Figure 1).

Levels of C4d and of C3d on erythrocytes

The mean number of C4d molecules per erythrocyte amongst the normal subjects was 209 (1 SE = 17), compared with 961 (SE 128) amongst the SLE patients without ACA ($z = 6.5$, $p<0.001$), and 1859 (1 SE = 562) amongst the SLE patients with ACA (ACA- vs ACA+, $z = 1.36$, $p = 0.09$). Similarly the mean number of C3d molecules amongst the normal subjects was 92 (1 SE = 10), amongst the SLE patients without ACA was 308 (1 SE = 51), and amongst the the SLE patients with ACA was 564 (1 SE = 134) (Figure 2). The C3d values were significantly different between each group (Figure 2).

Correlations between ACA levels and CR1 and complement on cells and in serum

The hypothesis was tested that raised levels of ACA were associated with reduced numbers of CR1 and with increased C3d and C4d deposited on erythrocytes. In Table 1 are shown correlations (and their significance values) between ACA levels (highest value

for IgM and IgG) and numbers of erythrocyte CR1, C4d, and C3d, and serum C4. A highly significant negative correlation was observed between ACA levels and CR1 numbers and a highly significant positive correlation between ACA levels and erythrocyte C3d numbers. These correlations remain significant even if corrected for the total number of correlations performed in this study (19). There were only very weak correlations observed between CR1 numbers and erythrocyte C3d and C4d levels (Table 1). These latter correlations did not alter when the analysis was restricted to those subjects with abnormally elevated deposition of C3d and C4d (> 2SD above the normal mean) on erythrocytes: CR1 versus C4d: RSp = -0.198 ($P > 0.05$); CR1 versus C3d: RSp = -0.177 ($P > 0.05$).

We then asked whether the effects of the IgM or IgG class of the ACA could be distinguished with respect to CR1 and complement levels on erythrocytes. Both IgG and IgM ACA levels were correlated inversely with CR1 numbers, and with each other (Table 2). However, whereas IgG ACA levels showed no correlation with erythrocyte C4d and C3d numbers, highly significant correlations were found between IgM ACA levels and these antigens.

<u>Correlations between DNAb and CR1 and complement on cells and in serum</u>

The relationships between levels of anti-DNA antibodies and erythrocyte CR1, C4d and C3d and serum C4 concentration were examined in order to test whether the observed correlations between ACA levels and these parameters could simply be explained by an association beween the levels of ACA and disease activity. There was no correlation between the levels of ACA and of DNAb (Table 1). Similarly there were no correlations between DNAb and erythrocyte CR1, C4d or C3d (Table 1). There was no correlation between ACA levels and serum C4 levels (Table 1), but in contrast, the DNAb was highly significantly negatively correlated with serum C4 levels (Table 1). This correlation remained significant when corrected for the number of tests performed.

Discussion

The factors controlling the numerical expression of CR1 on erythrocytes of patients with SLE are complex. Amongst normal subjects the level of CR1 expression correlates with two alleles identified by a restriction fragment polymorphism using a cDNA probe for the CR1 gene (6.9-kb allele associated with low expression and 7.4-kb with high expression) (4). These alleles also influence the levels of expression of CR1 amongst SLE patients. Patients homozygous for the 7.4-kb allele had higher CR1 numbers than individuals heterozygous for the 7.4- and 6.9-kb alleles, but, within each genotype, SLE patients were found to have lower CR1 numbers than their normal counterparts (5). This observation strongly suggests that, superimposed on this genetic control mechanism, there are other, as yet undefined, factors leading to reduced CR1 expression on erythrocytes of patients with SLE.

Observations that CR1 numbers were also low in patients with autoimmune haemolytic anaemias and in patients with paroxysmal nocturnal haemoglobinuria led to the development of the hypothesis that erythrocytes bearing autoantibodies or opsonic complement fragments may lose their CR1 by receptor proteolysis during the interaction of erythrocytes with cells of the fixed mononuclear phagocytic system (10). Studies showing that CR1 loss could be demonstrated on erythrocytes transfused into patients with SLE and / or haemolytic anaemias (22) supported the hypothesis that loss of CR1 from erythrocytes occurs within the circulation.

Two sets of observations on SLE patients suggested that antiphospholipid antibodies might be a factor associated with reduced CR1 levels on the erythrocytes of patients with SLE. The presence of antiphospholipid antibodies was correlated with positive direct antiglobulin reactions (15). Low CR1 numbers were similarly associated with positive direct antiglobulin reactions (23). The authors of this latter study postulated that the link

between low CR1 and the positive antiglobulin tests was the presence of immune complexes bound to erythrocyte CR1.

In the present study a correlation was detected between ACA levels and CR1 numbers on erythrocytes of SLE patients. This correlation was highly significant despite no account being taken of the subjects' CR1 numerical "genotype". Direct binding of ACA to erythrocytes has not yet been unequivocally shown. The strongest evidence was the observation of ACA activity in the eluate from erythrocytes of a single patient with high serum ACA levels and positive direct antiglobulin reaction (15). This and the present study suggest that either ACA or a closely related antibody bind to erythrocytes and may be related to low CR1 numbers.

IgM, but not IgG, ACA were also found to be correlated highly significantly with the number of C4d and C3d molecules on the patients' erythrocytes. Both IgG and IgM ACA levels correlated inversely with erythrocyte CR1 numbers, and it is hard to disentangle their relative contributions to the reduction of CR1. However it is noteworthy that there was only a very weak inverse correlation between CR1 numbers and levels of erythrocyte C4d and C3d in the present study. This observation implies that IgG ACA, which were not correlated with levels of complement deposition on erythrocytes, may be related to loss of CR1 by a mechanism independent of complement. An extension of the original hypothesis (10) to incorporate this finding would be that erythrocytes, opsonised with either IgG or complement, lose their CR1 on interaction with cells of the fixed mononuclear phagocytic system. The inclusion of many subjects with IgG ACA in the present study may explain the overall lack of correlation between CR1 levels and C4d and C3d numbers on erythrocytes, differing from previous observations (10).

In contrast to the studies of Norberg and colleagues (24) and Hazeltine and coworkers (15) we found no association between ACA and reduced levels of serum C4, although

we found the previously described inverse correlation between levels of anti-dsDNA antibodies and serum C4 levels (25). This may be explained by our studying different populations. Norberg and colleagues (24) compared C4 levels between patients with ACA and recurrent spontaneous abortions and women with recurrent spontaneous abortions and no antiphospholipid antibodies. Our population of patients with ACA comprised mainly patients with SLE, amongst whom many other autoantibodies which fix complement were present, eg anti-dsDNA antibodies.

The strength of the negative correlation (-0.43) between the level of ACA and mean erythrocyte CR1 numbers suggests that ACA may be an important acquired factor determining the reduction of erythrocyte CR1 in patients with SLE. Further studies are required in order to verify that ACA can bind directly to erythrocytes and that IgM ACA may fix complement to erythrocyte surfaces.

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Table 1.
Correlations between aCL level (highest reading of IgG or IgM), DNAb, and levels of erythrocyte CR1, C4d, C3d, and serum C4 levels.

Spearman Correlation	n	correlation	probability
aCL			
CR1 number	61	-0.43	**0.001**
erythrocyte C4d	61	0.33	**0.01**
erythrocyte C3d	61	0.41	**0.001**
serum C4	53	-0.09	0.533
DNAb			
CR1 number	57	0.15	0.27
erythrocyte C4d	57	0.08	0.58
erythrocyte C3d	57	0.16	0.24
serum C4	50	-0.43	**0.002**
CR1			
erythrocyte C4d	61	-0.22	0.092
erythrocyte C3d	61	-0.20	0.123
DNAb			
aCL	57	0.33	0.13

Table 1. Correlations between aCL level (highest reading of IgG or IgM), DNAb, and levels of erythrocyte CR1, C4d, C3d, and serum C4 levels.

Table 2.
Correlations between IgG and IgM aCL levels and erythrocyte CR1, C4d and C3d numbers.

Spearman Correlation	n	correlation	probability
IgG aCL			
CR1 number	61	-0.38	**0.002**
erythrocyte C4d	61	0.12	0.342
erythrocyte C3d	61	0.25	0.051
IgM aCL	61	0.37	**0.004**
IgM aCL			
CR1 number	61	-0.36	**0.004**
erythrocyte C4d	61	0.41	**0.001**
erythrocyte C3d	61	0.40	**0.002**
erythrocyte C4d			
erythrocyte C3d	61	0.73	**< 0.001**

Table 2. Correlations between IgG and IgM aCL levels and erythrocyte CR1, C4d and C3d numbers.

Figure 1 .

Mean number of CR1 molecules, expressed as molecules per erythrocyte, in 33 normal subjects, 34 SLE patients without anti-cardiolipin antibodies, and 27 SLE patients with anticardiolipin antibodies.

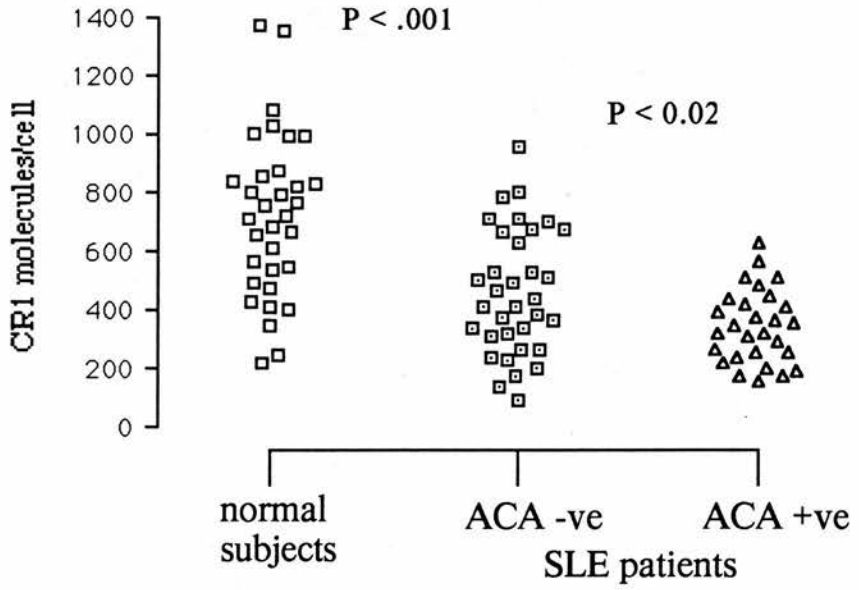

Figure 1. Mean number of CR1 molecules, expressed as molecules per erythrocyte, in 33 normal subjects, 34 SLE patients without anticardiolipin antibodies, and 27 SLE patients with anticardiolipin antibodies. The number of CR1 molecules per red cell amongst the aCL-negative SLE patients was significantly lower than amongst the normal subjects ($z = 3.77$, $p < 0.001$). Similarly the mean CR1 number was significantly lower amongst the aCL-positive SLE patients compared with their aCL-negative counterparts ($z = 2.34$, $p < 0.02$).

Figure 2.

Mean number of C3d molecules, expressed as molecules per erythrocyte, in 33 normal subjects, 34 SLE patients without anticardiolipin antibodies, and 27 SLE patients with anticardiolipin antibodies.

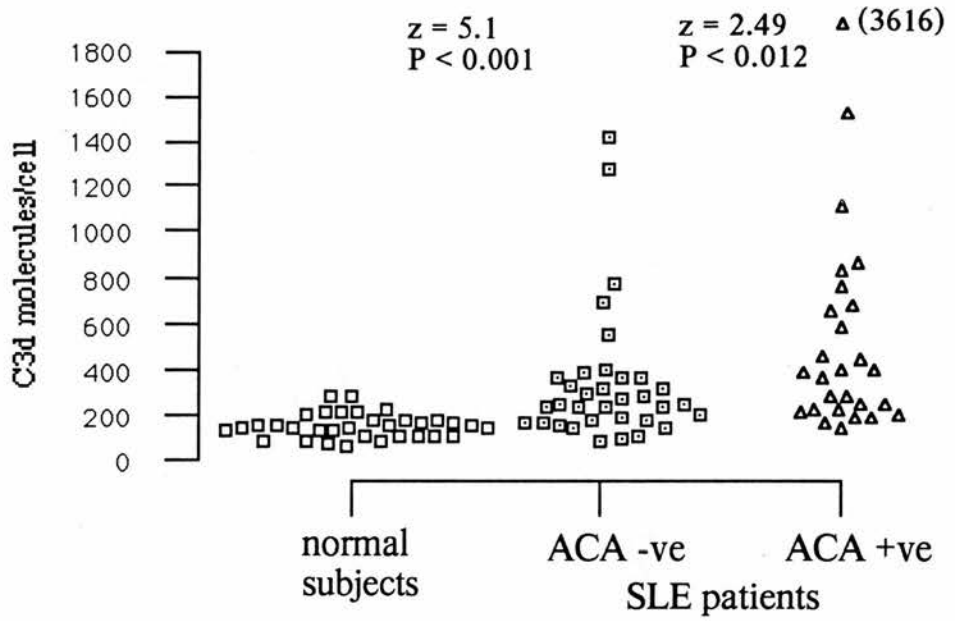

Figure 2: Mean number of C3d molecules, expressed as molecules per erythrocyte, in 33 normal subjects, 34 SLE patients without anticardiolipin antibodies, and 27 SLE patients with anticardiolipin antibodies. The number of C3d molecules per red cell amongst the aCL-negative SLE patients was significantly higher than amongst the normal subjects ($z = 5.1$, $p < 0.001$). Similarly the mean C3d number was significantly higher amongst the aCL-positive SLE patients compared with their aCL-negative counterparts ($z = 2.49$, $p < 0.012$).